Production of Bioethanol from Guinea Grass (*Panicum maximum)* and Ragi Tempeh as Fermentation Starter using Bioreactor and Shaker

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DECLARATION

I hereby declare that the work embodied in this report is the result of the original research and has not been submitted for a higher degree to any universities or institutions.

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I certify that the report of this final year project entitled "Production of Bioethanol from Guinea Grass *Panicum maximum* by Using Ragi Tempeh as Fermentation Starter through Fermentation in Bioreactor and Shaker" by Lam Zheng Kang, matric number F15A0067 has been examined and all the correction recommended by examiners have been done for the degree of Bachelor of Applied Science (Bioindustrial Technology) with honours, Faculty Bioengineering and Technology, Universiti Malaysia Kelantan.

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LIST OF ABBREVIATIONS AND SYMBOLS

NO_X	Nitrogen oxide
v/v	Volume/volume percent
HPLC	High Performance Liquid Chromatography
FTIR	Fourier Transform Infrared Spectroscopy
CO_2	Carbon dioxide
LAB	Lactic Acid Bacteria
β	Beta
SSF	Simultaneous Scarification and Fermentation
SHF	Separate Hydrolysis and Fermentation
g	Gram
UV	Ultraviolet
VIS	Visible light
PDA	Photodiode Array
RI	Refractive Index
$AgNO_3$	Silver Nitrate
Ag_2O	Silver Oxide
$NaNO_3$	Sodium Nitrate
H_2O	Water
$[Ag(NH_3)_2]^+$	Diammninesilver (I) complex
NH_3	Ammonia
H_2SO_4	Sulphuric acid
NaOH	Sodium Hydroxide
KH_2PO_4	Potassium dihydrogen phosphate
g/L	Gram per litre
w/v	Weight/volume percent
mL	milliters
RPM	Revolution per minute
M	Mole
IR	Infrared Ray
LC	Liquid Chromatography

mL/min	Millilitre per minute
µl	Microlitre
nm	Nanometer
cm^{-1}	Reciprocal wavelength
R^2	coefficient of determination
min	minute
µV	microvolts
ACN	Acetonitrile

Production of Bioethanol from Guinea Grass *Panicum maximum* and Ragi Tempeh as Fermentation Starter using Bioreactor and Shaker

Abstract

Nowadays, the depletion of fossil fuels as prime source of energy is a serious issue that trigger the research and development of biofuel. Biofuel as renewable fuel source is sustainable and less carbon emission to environment compare to fossil fuels. Currently, the first- and second-generation biofuel has high potential to be commercialise, especially second-generation biofuel that using lignocellulosic feedstock as material that would not compete with edible food crops. This study was investigating the production of bioethanol from the mixture of guinea grass (Panicum maximum) and using ragi tempeh through fermentation. The acid pretreatment process was carried out using 15% v/v sulphuric acid, it is considered as concentrated acid pretreatment and the reason for selecting this method is because it release higher amount of glucose compare with diluted acid pretreatment process, with concentration of 0.6%, 0.9%, 1.2%, 1.5%, and 5.0% at most. The fermentation was carried out in anaerobic condition using bioreactor and shaker at 37°C and different pH of 5,6,7, and 8. Freezing test and Tollens' test result showed very ethanol concentration in the sample product is very low, and aldehyde compounds, which is the inhibitory substance for microorganisms' activity during ethanoic fermentation is present in product sample. The aldehyde is releases due to degradation of lignocellulosic feedstock by acid hydrolysis. FTIR and HPLC is carried out for getting more accurate result. FTIR result showed the major component of recovered sample is water, beside water the major component for the sample wasn't ethanol, it is suspected to be lactic acid, glucose, or aldehyde. The best result that showed higher ethanol yield is 7.89%, obtained by fermentation using shaker at pH 8 for 48 hours.

Keywords: Bioethanol, ragi tempeh, *Panicum maximum*, acid pretreatment, bioreactor

Penghasilan bioetanol dari guinea grass (Panicum maximum) dan ragi tempeh sebagai pemula fermentasi melalui bioreaktor dan shaker

Abstrak

Pada masa kini, kukurangan bahan api fosil sebagai sumber tenaga yang berharga adalah salah satu isu pencetusan bagi penyelidikan dan pembangunan bahan api bio. Bahan api bio sebagai sumber bahan api yang boleh diperbaharui mempunyai sifat kelestarian dan pelepasan karbon kepada alam sektiar adalah lebih kurang berbanding dengan bahan api fosil. Pada masa kini, bahan api bio generasi pertama dan kedua mempunyai potensi tinggi untuk dikomersilkan, terutamanya bahan api bio yang menggunakan bahan mentah lignoselulosa sebagai bahan yang tidak akan bersaing dengan tanaman makan yang menjadi bahan makanana utama kepada manusia. Kajian ini digunakan untuk menyiasat pengeluaran bioetanol dari rumput guinea (*Panicum maximum*) dengan menggunakan ragi tempeh sebagai pemula fermentasi yang digunakan dalam process fermentasi. Penapaian ini dilakukan dalam keadaan anaerobik menggunakan bioreaktor dan shaker pada 37 ° C dan pH yang berbeza 5,6,7, dan 8. Ujian pembekuan dan keputusan ujian Tollens menunjukkan kepekatan sangat etanol dalam produk sampel sangat rendah, dan aldehida sebatian, yang merupakan bahan penghalang untuk aktiviti mikroorganisma semasa penapaian etanoik terdapat dalam sampel produk. Aldehida dikeluarkan disebabkan oleh degradasi bahan mentah lignoselulosa oleh hidrolisis asid. FTIR dan HPLC dijalankan untuk mendapatkan hasil yang lebih tepat. Hasil FTIR menunjukkan komponen utama sampel yang pulih adalah air, di samping air komponen utama untuk sampel itu bukan etanol, ia disyaki adalah asid laktik, glukosa, atau aldehida. Keputusan terbaik yang menunjukkan hasil etanol yang lebih tinggi ialah 7.89%, diperolehi dengan penapaian menggunakan shaker pada pH 8 selama 48 jam.

Kata kunci: Bioethanol, ragi tempe, *Panicum maximum*, pretreatment asid, bioreaktor

CHAPTER 1

INTRODUCTION

1.1 Background of Study

The fossil fuels depletion such as natural gas, petroleum, coal, and other, which is the precious sources of energy in the current century is highly concerned by researcher, some researcher believe the source will be collapse in future. Finding of biofuel was solution for substitution of fossil fuel as energy source. Among the kind of biofuel choice, bioethanol is the one of the best choice. Compare to fossil fuel, combustion of bioethanol is cleaner, less carbon emission to atmosphere, and have potential to reduce particulate and NO_X (nitrogen oxide) emissions in compression-ignition engines. The use of ethanol was the blended gasoline in the concentration range of 10-85% (v/v), depends on the quality of biofuel. (Vohra, et al, 2014) It is because bioethanol has high octane number and low cetane number. High octane number represents fuels resists spontaneous ignition and have less tendency to knock in a gasoline engine, which is preferable; higher cetane number means the fuels ignite readily and therefore perform better in diesel engine. Example of blend of bioethanol for vehicles is E85 fuel, which contain 85% bioethanol and 15% gasoline. In tropical regions country like India, Brazil, and Colombia, the sugarcane is main feedstock used for ethanol fuel production because it is in tropical

region, while other country like United States, European Union, and China, used the corn as dominant feedstock for ethanol fuel production. (Vohra et al., 2014). The comparison of feedstock used, product, pros and cons, and others among petroleum refinery, 1st generation fuel, 2nd generation is showed in figure 1.1.

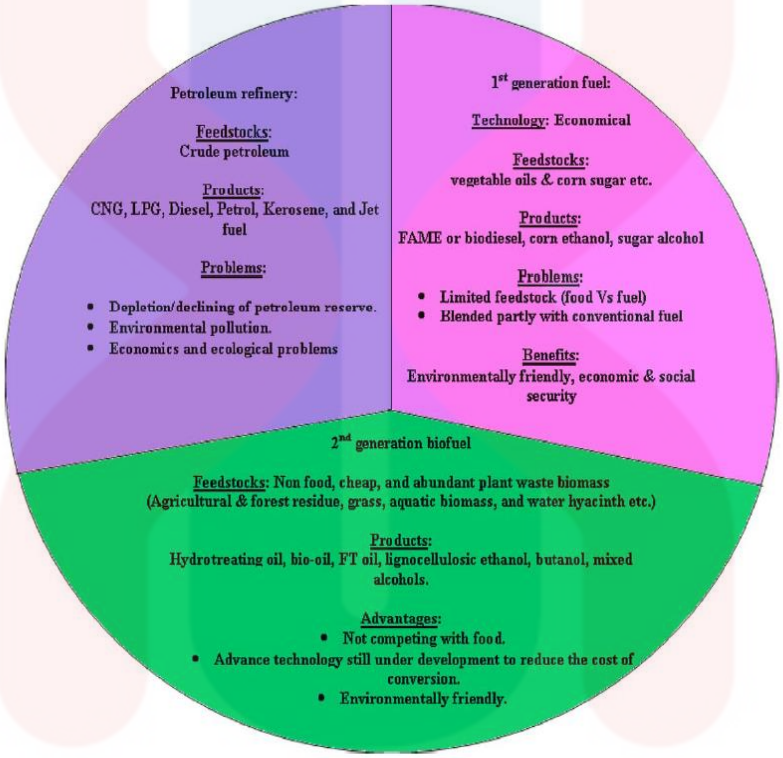

Figure 1.1: Comparison of petroleum fuel with first and second generation biofuel.

Source: Vohra et al. (2014)

The study of biofuel can be divided into 4 generation, the research of bioethanol in this study was belongs to second generation. The development of first generation biofuel was using the oil crop such as oil palm as material source. Triglyceride feedstock derived from plant or animal sources generally consist of fatty acids and glycerol. The corn and sugarcane was the most preferable choice because it is easy to produce sugar monomer by hydrolysing the polysaccharides (Vohra et al., 2014). Although first generation biofuel was a breakthrough of alternative fuel source research, but researcher

found several drawback that trigger the research of second generation biofuel. Launching of first generation biofuel must contribute to raise of worldwide food price due to competition with food crops. Although biofuel was a cleaner fuel source, but its production bring negative impact to the environment (Hassan & Kalam, 2013). First generation biofuel is expensive and will accelerating deforestation due to indirect land use effect, which will damage the biodiversity and natural.

The research of bioethanol production has developed into second generation due to those limitation associated with first generation biofuel. Researcher use second generation feedstock, such as agricultural waste, forest residue, municipal solid waste, green waste and non-food crop feedstocks as source of material, also categorised as non-edible lignocellulosic biomass. Those residue mainly composed of non-food part of crops, such as stems, leaves, husks, and others. The second generation routes used to produce biofuel was thermochemical routes, biochemical routes, and oleochemical processes. (Naik et al., 2010) The biochemical route is using enzyme and other micro-organisms to convert cellulose, hemicellulose component of the feedstock to sugars prior to fermentation process; thermochemical process is using mainly pyrolysis or gasification technologies to produce synthesis gas which a wide range of long carbon chain biofuel can be reform; oleochemical processes is the hydroprocessing of lipid feedstock obtained from oilseed crops and algae. The research on second generation biofuel was ongoing and relatively immature, although first generation biofuel has limitation but it will continue launched to fulfilled current demand, and improve at the same time as fundamental of second generation biofuel before its technologies and fully commercialise.

Involving of pre-treatment and enzymatic hydrolysis process causing the production of second-generation biofuel become very costly; the source of agriculture/municipal waste used as material was not sustainable too. Researcher continue

to develop third generation biofuel and feedstock, algae was selected because of its high growth rate and harvesting cycle, with minimal nutrient level unless required economical production. Algae is the core third generation biofuel research and development process. Previously, algae were pick as raw material for second generation biofuel production, scientist founded it has higher energy yield and more accessible than other second-generation biofuel feedstock material. The term algae included microalgae, macroalgae, and cyanobacteria, meanwhile algae oil is general term of third generation biofuel product, it can range from ethanol, biodiesel, syngas, bio-oil and other chemical feedstock. (Jim Bowyer et al., 2018) Current status of third generation biofuel production is immature and mostly still proceeding in laboratory stage to find out better harvesting technique, biorefinery concept, photobioreactor design, downstream processing and others. Therefore, some researcher has started the fourth generation biofuel research, which is using bio-engineering technique to enhance yield of production, improve feedstock quality, and genetic modification. Unlike third generation algae oil, fourth generation biofuel involve the modification of biogenic feedstock using bio-engineering and biotechnology skill. Research of fourth generation including the breeding of new species algae or cyanobacteria for quality optimization of third and fourth generation biofuel.

Plant biomass belongs to renewable source of energy that provided many advantage of research because it is clean and environment friendly, switchgrass (*Panicum virgatum L.*) and elephant grass was example of energy crops that used to harvest bioethanol. The *Panicum maximum* (guinea grass) has high potential to use as substitution of energy source because of its high production yield and optimal seed propagation. Guinea grass is the common name of *Panicum maximum*. Those roadside and untended area showed high distribution of the *Panicum maximum* colony, it is showing high potential on forms clumps and foster erosion. The population and survivability of

Panicum maximum was strong because it is leafy, high production potential, drought tolerant, and early season growth in certain area. Its seed can be easily spread along water courses and unglazed roadsides and is the major weed in sugar cane field due to shaded conditions in field.

In this research project, the *Panicum maximum* was used as lignocellulosic biomass because it is easily accessible. Other than *Panicum maximum,* the *Panicum virgatum,* (switchgrass) is now used as feedstock material for second generation biofuel production. *Paniucm virgatum* has incredible survival tolerance to various growing season, land condition, soil, and weather condition, its habitat was focus on North America like Canada, United States, and Mexico. *Panicum maximum* and *Panicum virgatum* was both energy crops, if the *Panicum virgatum* can used for bioethanol production, then *Panicum maximum* have high potential to be selected as feedstock material for bioethanol too.

1.2 Problem Statement

For biochemical conversion pathway in bioethanol production, selection of feedstock material and fermentation starter is important. Second generation prefer the use of unwanted feedstock or agriculture waste. Unless there is a systematic way to collect agriculture waste, or else the source of agriculture waste is not stable and will create competition with that agriculture product if the add-value of waste is greater than the agriculture product itself. The agriculture waste such as potato strips, orange peels, and others has high potential for ethanol production, but it is hard to collect and required screening and processing to remove impurities from the desire waste itself. Compare to

collecting agriculture residue, the used or cultivation of energy crops in natural such as *Panicum maximum*. The fermentation starter is the starter culture that involve microorganisms and medium such as nutrient liquid or other media like grains, that is colonized by the microbes. The famous fermentation starter was yeast, ragi, koji and others.

In this research, the use of *Panicum maximum* and ragi tempeh is the combination with potential for bioethanol production. As energy crops, *Panicum maximum* has high energy yield for bioethanol production, hence the selection of fermentation starter will affect the yield of bioethanol conversion from glucose. Ragi tempeh is the fermentation starter used for tempeh production, a traditional soy product came from Indonesia. The performance of ragi tempeh on fermentation of soy is good, but its performance on bioethanol production from lignocellulosic feedstock is waited to be research.

In this study the bioethanol production will be carry out using bioreactor and shaker. The most significant difference between the use of bioreactor and shaker is control of fermentation condition and the production yield. In general, using bioreactor will have better control of system to manipulated fermentation condition, but shaker fermentation is faster and good in quantitative production. This study will find out the performance different between bioreactor and shaker using guinea grass and ragi tempeh.

1.3 Objectives

1. To investigate the possibility of using Panicum maximum for bioethanol production using ragi tempeh as fermentation starter.
2. To characterize the chemical composition of the bioethanol produced from *Panicum maximum*.
3. To determine the effect of different factors such fermentation pH and acid concentration during pretreatment for ethanol production

1.4 Scope of Study

The scope of study is to determine the possibility of bioethanol production form *Panicum maximum* using ragi tempeh as fermentation starter. The popular lignocellulosic biomass that flavour by researcher for development of biofuel production more likely to agriculture waste, so the possibility of using grass as bioenergy source is required further research and development. Currently the popular grass used for biofuel production was switchgrass, and there is lots of case study on using switchgrass as feedstock ethanol in United States, such as the case study located in East Tennessee. (Burton et al., 2013) In this research paper it is focused on biochemical conversion pathway, which is fermentation but not thermochemical pathway such as pyrolysis, gasification and others. This is the decision after evaluate current equipment and facility available in UMK that can support this research project. The production yield was evaluate using several variable such as concentration of acid during chemical pretreatment and different pH of fermentation broth. Those parameter was focus on 2 important procedure of bioethanol production, which is pretreatment and fermentation, to identify its significant different

and explore the variable provide optimal production yield and analysis. After the product ethanol was recover from the fermentation broth, it is require to carry out quantitative and qualitative analysis by High Performance Liquid Chromatography (HPLC) and Fourier Transform Infrared Spectroscopy (FTIR).

1.5 Significant of Study

The production of biofuel or synthetic chemical using first generation crops has high achievement since last decade, Over 45% of the world's bio-chemicals were being produced by fermentation during the year 2006, according to a survey conducted by SRI Consulting. (Biorenewable chemicals: Feedstocks, technologies and the conflict with food production). Bioethanol is getting more significant as alternative fuel source for replacement of the gasoline. The use of second generation bioethanol can improve CO_2 balance, if it can be produced using unwanted material for its production it is more desired as it doesn't conflict with edible crops substrate. Due to absent of inexpensive technologies and skill for the utilization of algal and lignocellulosic biomass to lower cost biofuel, many manufacture of biofuel continue the production of high value chemical and fuel through old-traditional technique, ignoring the fact that competition of edible crop as feedstock will bring impact to worldwide food market and global hunger issue.

Research of switching the choice of feedstock from first generation feedstock which is edible feedstock to second generation feedstock, which is non-edible feedstock is urgent and highly recommended. Some company of research institution start to investigate more economical methods, in order to promote second generation biofuel production from laboratory or pilot scale to mass production. From the view of

socioeconomic, using grass as lignocellulosic feedstock was better and add-value to grass. Same as using agriculture or municipal waste, successfully produce biofuel from grass can avoid food crisis issue and find alternative for cheaper alternative fuel production.

CHAPTER 2

LITERATURE REVIEW

2.1 Background of *Panicum maximum*

Panicum, or we called panicgrass is a large genus of grasses, 450 annual and perennial species native throughout the tropical region of the world. The example of Panicum grasses was *P.coloratum*, *P.infestum*, *P.maximum*, *P.phragmitoides*, and *P.turgidum*. The *Panicum maximum* is considered to be valuable, it has high leaf and seed production which is very palatable to livestock. This grass was easily to growth and has high drought-tolerant ability (Ammondt & Litton, 2012). The *Panicum maximum* are preferable as energy crops for second generation ethanol production because it fulfilled the requirement on biomass selection, which is high cellulose concentration and low lignin formation (Naik et al., 2010).

Panicum maximum is famous tropical grass and easy to found in tropical country like Malaysia, it is easily to growth everywhere because it require adequate water to growth, and is persistent weed in cultivated areas as it only required very little maintenance to survive. Once it is mature it is hard to remove. Seed production in *P. maximum* is problematic due to the extended flowering period, combined with shattering of the seeds (Ammondt & Litton, 2012).

2.2 Ragi Tempeh and Fermentation

Ragi tempeh, also known as tempeh starter, is very essential in tempeh fermentation process. The starter culture in the tempeh starter was *Rhizopus oryzae*, *Rhizopus oligosporus* and other. In Indonesia, *Rhizopus spp* was famous and play role as inoculum source for making tempeh. *Rhizopus oligosporus* and *Rhizopus oryzae* plays important role as main microorganisms in fermentation process. Other microorganisms other than fungi might able to use for tempeh fermentation. Using *Rhizopus spp* bacteria for ethanoic fermentation have its potential, the hexoses and pentoses released from pretreatment can be assimilated by *R. oryzae* for bioethanol production, this microbes also can growth in environment of higher temperature compare with *S. cerevisiae*, chitosan is the valuable by-products obtain from its biomass (Abedinifar, Karimi, Khanahmadi & Taherzadeh 2009). The lactic acid bacteria (LAB) is present during tempeh fermentation, researchers founded the LAB is present in tempeh and suggested that during tempeh fermentation, the presence of high number of LAB during tempeh fermentation suggested that there was interaction between mould as main organisms for tempeh fermentation and yeast that also present at high number (Azmi, Yusuf, Jimat, & Puad, 2016). LAB may obtain nutrients from moulds or yeasts metabolism, it could hydrolyse soy protein, but the yield was much less than mould and yeast. (Nurdini et al., 2014). In food fermentation, yeast is known to grow in synergism with LAB, it is able to synthesize vitamins, amino acids and purines, also breaks down carbohydrates complex which is essential for the LAB growth (Azmi et al., 2016). Meanwhile, yeast could consume lactic acid that produced by LAB (Azmi et al., 2016).

Using ragi tempeh for fermentation process is estimated to produce ethanol and lactic acid as product of fermentation. The presence of lactic acid bacteria and Rhizopus bacteria showed heterofermentation properties, the glucose is consume and produce lactic acid and ethanol (Bott, 2014). *R. oryzae* was able to grow on the hydrolyzates and produce ethanol (Abedinifar et al., 2009). The Cultivation of *R. oryzae* in a nitrogen-limited medium resulted in 78% of the theoretical yield of lactic acid, and the major by-products were glycerol, ethanol, and fumaric acid, whereas xylose fermentation yielded 70% lactic acid with ethanol and glycerol as by-products (Millati, Edebo, & Taherzadeh, 2005).

2.3 Lignocellulosic Biomass

Energy crops have high yield of lignocellulosic biomass and suitable to be the raw material for second generation bioethanol production. The three main organic compounds of cell wall was cellulose, hemicellulose and lignin. Understand these component of lignocellulosic materials giving much information on processing lignocellulosic material for bioethanol production. The most important component in the plant cell wall is cellulose, and glucose is the main substrate for biochemical processing of biofuel production. Hence the depolymerisation process of lignocellulosic material is important. Current challenge for second generation biofuel production is absent of low-cost skill or technology for process those material. From the view of economic, utilize of lignocellulosic biomass has advantage of avoiding edible plant competition in food market such as corn starch and sugarcane.

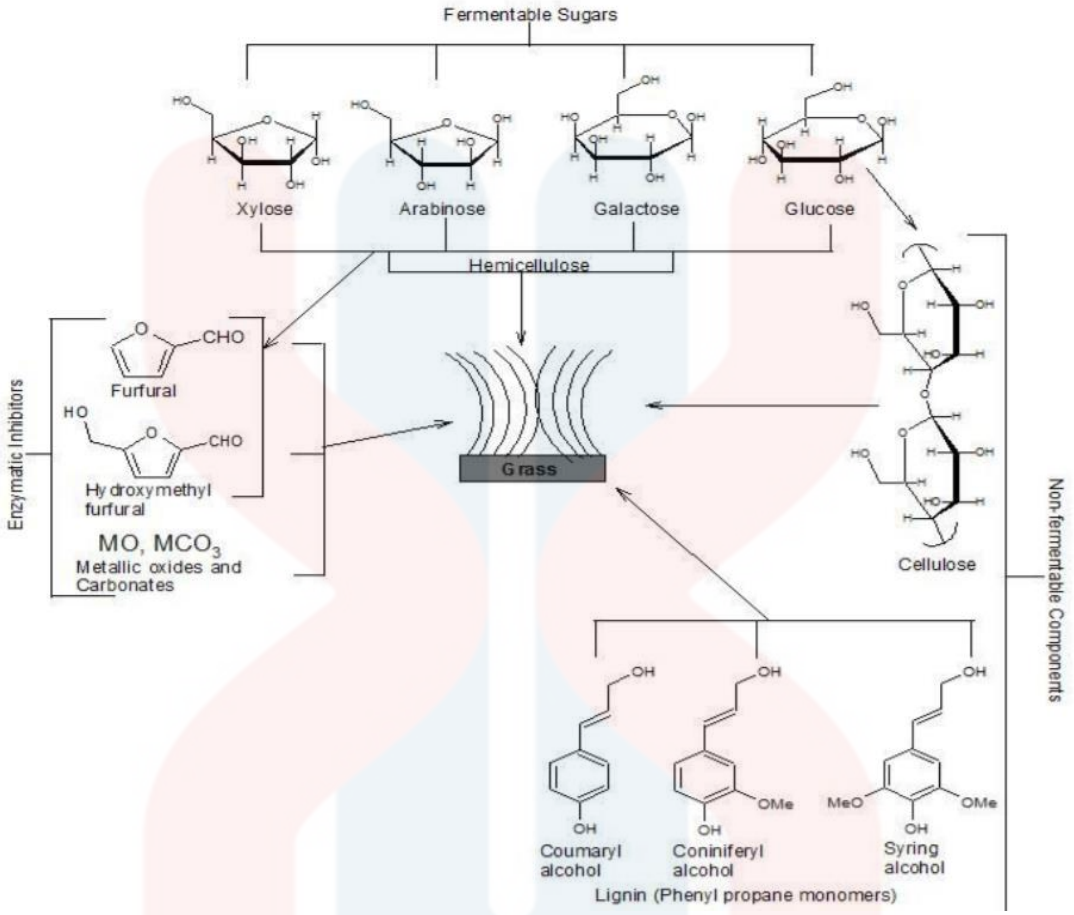

Figure 2.1: The fermentable and non-fermentable components in a grass with the component that can cause recalcitrant effect to hydrolysis and fermentation.

Source: Ofodile (2016).

Biomass composition is important in ethanoic fermentation. Essential process for bioethanol production was pretreatment of lignocellulosic biomass to gain polysaccharides, and scarification process to gain fermentable monosaccharide. The composition of lignocellulosic biomass in *Panicum maximum* was: 32.57% of hemicellulose, 32.71% of cellulose, and 3.09% of lignin (Ofodile, 2016). The source of lignocellulosic biomass would affect its component. The example of lignocellulosic biomass material was: softwood, hardwood, grasses, municipal waste, and others. The following table will show those main component of those lignocellulosic material, high cellulose and low lignin of lignocellulosic material is the preferable material.

Table 2.1: Percent composition of lignocellulosic components in various lignocellulosic materials.

Lignocellulosic material	Lignin (%)	Hemicellulose (%)	Cellulose (%)
Sugar cane bagasse	20	25	42
Sweet sorghum	21	27	45
Hardwood	18 – 25	24 – 40	40 – 55
Softwood	25 – 35	25 – 35	45 – 50
Corn cobs	15	24	45
Corn stover	19	26	38
Rice straw	18	24	32.1
Nut shells	30 – 40	25 – 30	20 – 30
newspaper	18- 30	25 – 40	40 – 55
Grasses	10 - 30	25 – 50	25 – 40
Wheat straw	16 – 21	26 – 32	29 – 35
Banana waste	14	14.8	13.2
bagasse	23.33	16.52	54.87
Sponge gourd fibres	15.46	17.44	66.59

Source: Shahzadi et al. (2014).

2.3.1 Cellulose

The most abundant and renewable organic composition in lignocellulosic biomass is cellulose, also it the most significant component for biofuel processing. Cellulose is polysaccharide composed of β-1,4-glycosidic bonds, and formed by carbon (44.44 %), hydrogen (6.17 %), and oxygen (49.39 %). (Amin et al., 2017) The degree of polymerisation (DP) for cellulose can be range from hundred to ten thousand number of glucose group, $(C_6H_{10}O_5)n$. From view of physical, cellulose has compact and high stability structure because of the hydrogen bond of the cellulose, make it hard to be attack or break down unless using enzyme or other specific method.

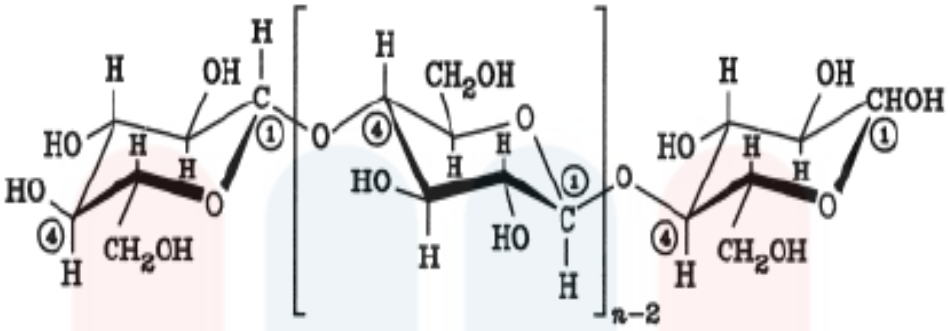

Figure 2.2: structure of single cellular molecules

Source: Harmsen, Huijgen, López, & Bakker. (2010).

The degradation of cellulose is an important reaction that can be used to produce cellulose products. Acid degradation, microbial degradation, and alkaline degradation are mainly to break the glycosidic bonds between two adjacent glucose molecules; an alkali peeling reaction and oxidation-reduction reaction of cellulose usually act on reducing ends of celluloses, and the oxidative degradation of the cellulose occurs mainly in dissociating hydroxyls at C2, C3, and C6 of the glucosyl ring (Harmsen et al., 2010). Cellulose molecule chains will form carbonyls at C2 when oxidized to some degree and then be degraded in the following alkali treatment process by the elimination reaction of β-alkoxy. After disconnecting the glycosidic bond, the reaction product is formed and then degraded to a series of organic acids. Cellulose is the most essential biopolymer for ethanoic fermentation, the higher the amount of cellulose content, the higher the yield of bioethanol.

2.3.2 Hemicellulose

Hemicellulose is made up of many types of subunit such as D-Xylose, mannose, L-arabinose, galactose, glucuronic acid and others. Similar to cellulose, the bonds between the subunit consist of β-1,4-lycosidic bonds in main chains, but its side chain have β-1.2-, β-1.3-, and β-1.6-glycosidic bonds in side chains (Amin et al., 2017). Among others substance, hemicellulose have very less glucose group in polymerisation, approximate less than 200, because of this low degree of polymerisation, it is more easily to degrade. It is generally believed that hemicellulose is the glucan in the matrix of the cell, and the main component are xylan, xyloglucan, glucomannan, manna, galactomannan, and others (Amin et al., 2017). Hemicellulose is insoluble in water unless the temperature is high, acid hydrolysis is suitable pretreatment process to break down the structure of hemicellulose. After pretreatment, the pentose and hexose will release from hemicellulose, but the process for ferment this 2 sugar for bioethanol production is complicated, if using traditional enzyme. Beside hexose which is easy to ferment, pentose require specific engineered enzyme for hydrolysis and ferment. So it is not recommended to use for ethanoic fermentation. Pretreatment process can remove hemicellulose, reduce cellulose crystallinity, and increase porosity of the material. (Sun & Cheng, 2005)

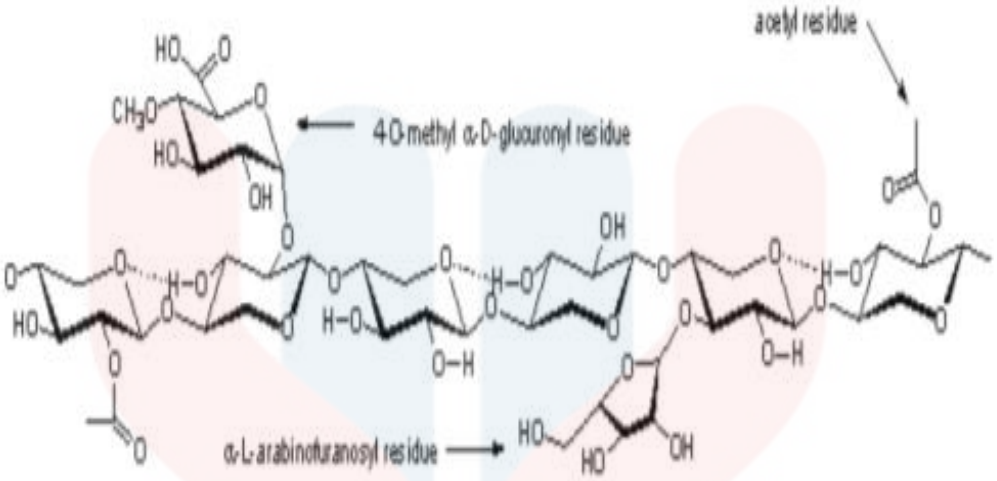

Figure 2.3: A schematic representation of the hemicellulose backbone of arborescent plants.

Source: Harmsen et al. (2010).

2.3.3 Lignin

Lignin is the most abundant organic component in plant, just like cellulose, but it represent approximate 10% to 25% of the lignocellulosic biomass. (Amin et al., 2017) Lignin used to filling the gap between the cellulose and hemicellulose, from view of physical it is act as protection layer against cellulosic and hemicellulose. Lignin is a polyphenolic polymer with a three-dimensional network. Lignin is a complicated amorphous polymer with three-dimensional network, it includes three basic structural monomers: p-phenyl monomer (H type) derived from coumaryl alcohol, guaiacyl monomer (G type) derived from coniferyl alcohol, and syringyl monomer (S type) derived from sinapyl alcohol. (Amin et al., 2017). Because of these three types of monomers, lignin cannot provide the carbohydrate for biofuel production, removal of lignin from biomass will enhance the ethanol yield form fermentation.

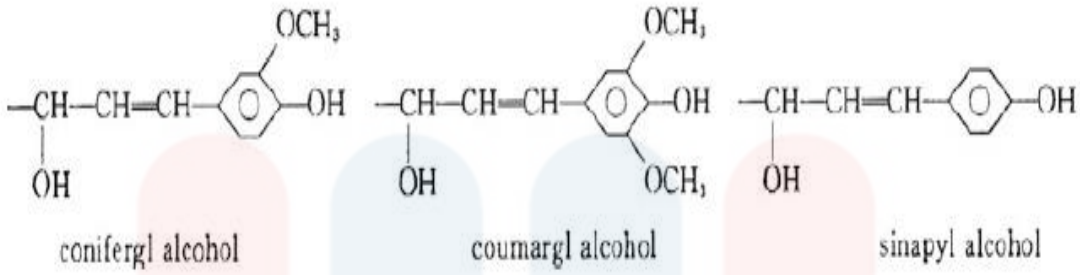

Figure 2.4: P-coumaryl- , coniferyl- and sinapyl alcohol: dominant building blocks of the three- dimensional polymer lignin.

Source: Harmsen et al. (2010).

2.4 Biofuel

2.4.1 First and Second Generation Biofuel

In current decade, Brazilian sugarcane ethanol is regarded by some specialist as the only biofuel currently produced at commercial scale to meet advanced non-cellulosic renewable fuel target. (Aro, 2016) In general, the first generation ethanol involve technology fermentation, distillation; and hydrolysis of starch. Second generation biofuel have similar production technology but focus on hydrolysis and fermentation. Main product for generation 1 biofuel was bioethanol and biofuel, bioethanol was produced by fermentation of sugar from crops; biodiesel is produced through trans-esterification of vegetable/animal fat with alcohol to produce fatty acid methyl esters. Bioethanol is used as fuel additive in gasoline at roughly 10% rather than a total replacement, biodiesel has same function as fossil fuel diesel but fossil fuel-based diesel Is hydrocarbon consist of 12-20 carbon atoms, whereas biodiesel is a three-carbon ester that burn much like diesel. (Araújo, Devinder Mahajan, Kerr, & Silva, 2017)

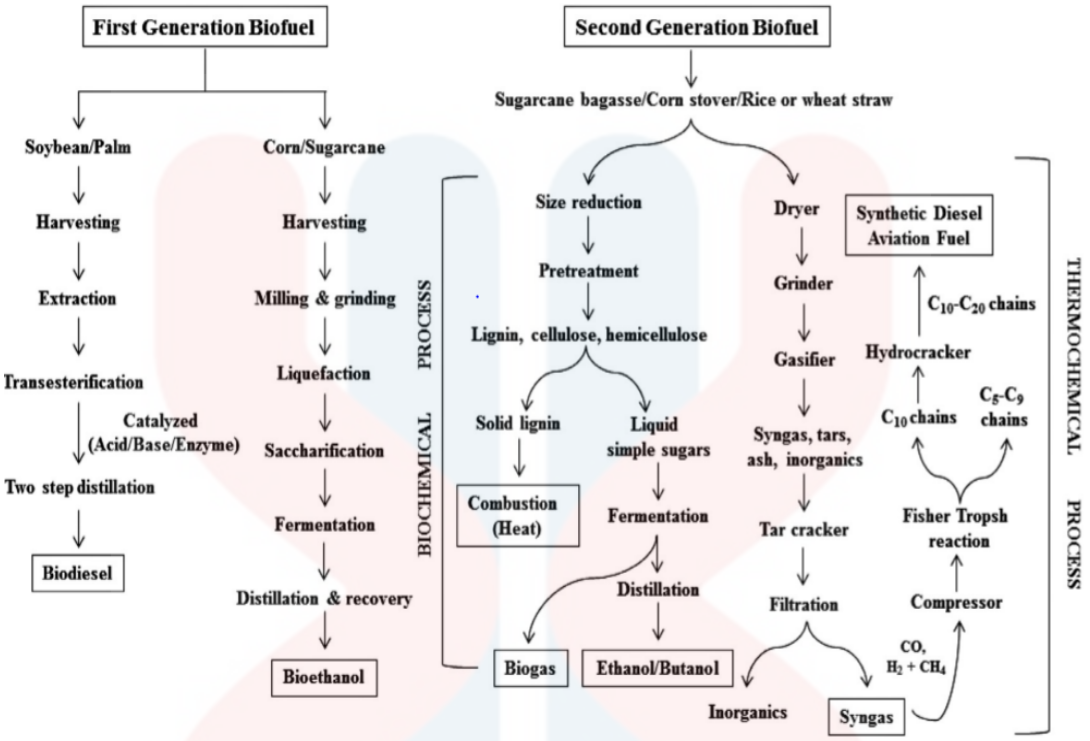

Figure 2.5: Technologies involved for production of first and second generation of biofuel.

Source: Dutta, Daverey, & Lin. (2014).

Second generation biofuel using biochemical and thermochemical pathway, it include bioenergy crops and inedible parts of ordinary crops and forest trees. The selection of plant/feedstock that have good potential for biofuel production depends on some few trait: photosynthesis, stress tolerant, nutrient uptake, and water use efficiency. After selection for suitable feedstock, researcher was focused to enhance all traits mentioned above for optimise biofuel yield for large scale production. (Aro, 2016) The success in the commercial development and development of second generation biofuel technologies will required significant progress in a number of areas if the technological and cost barriers they currently face to be overcome.

Nowadays, researcher tried to Improved understand of currently available feedstock, using experience in the production of various dedicated feedstock such as switchgrass to understand or estimated its yields, characteristic and costs. From the view of technology, the pre-treatment technology for second biofuel production is still inefficient but costly. Beside pre-treatment process, use of enzyme for processing also very expensive and have the risk to produce product inhibitor. For optimum yield, the best condition of manufacture was convert all C5&C6 sugars into ethanol; the presence of inhibitor such as furfural, hydroxyl methyl furfural, or natural wood-derived inhibitors will reduce the yield for bioethanol production. Hence technology and understanding on second biofuel production should start from the fermentation yield viewpoint.

2.4.2 Third and Fourth Generation Biofuel

Third generation biofuel was produced from microalgae and microbes that don't compete with feedstock of human and animal. Mircoalgae are single-cell microscopic organisms which are naturally found in fresh water and marine environment, it has high growth rate, high production capacity of lipid, and fixation of greenhouse gaseous, it can provide several different types of renewable biofuels, includes methane, biodiesel, and bio-hydrogen. Several algal species such as *Botryococcus braunni*, *Chaetocero scalcitrans*, several Chlorella species, *Isochrysis galbana*, *Nanochloropsis*, *Schizochytrium limacinum* and Scenedesmus species have been studied as potential sources of biofuel. (Dutta et al., 2014) The pathway for converting microalgae biomass into energy sources include biochemical conversion, chemical reaction, and thermochemical conversion (Alam, Mobin, & Chowdhury, 2015) Biofuel production from algae biomass can be commercially viable if algal by-products are optimally utilised.

The oil part of algae biomass is around 30% and the remaining 70% is algae by-product. This by-product can be utilised as nutrients for feedstock, pharmaceutical ingredients, cosmetics, toiletries and fragrance products. (Alam et al., 2015)

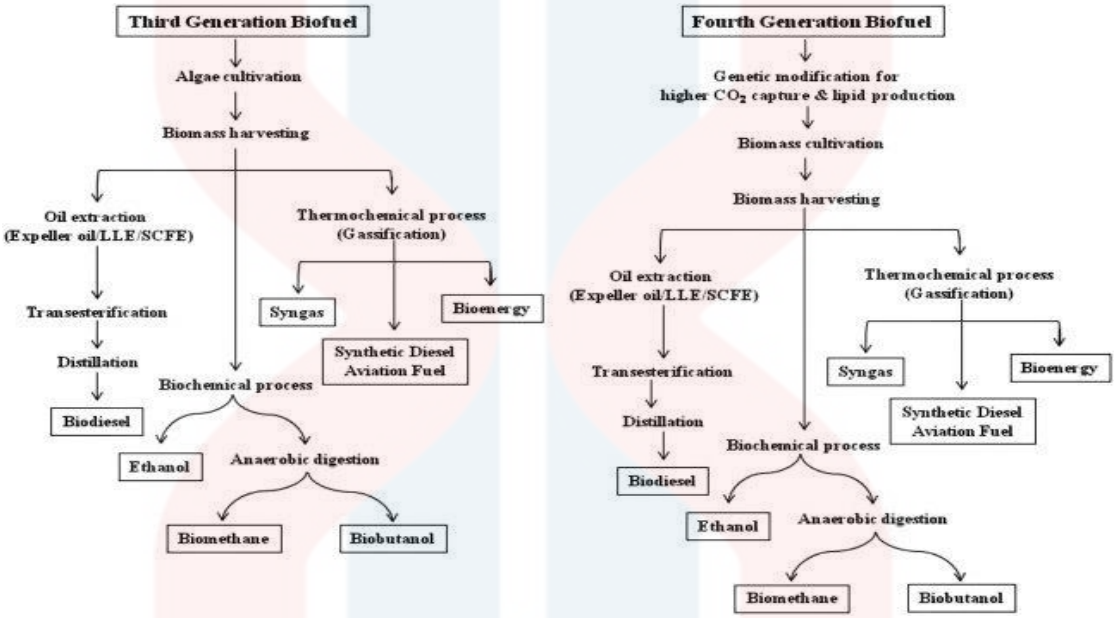

Figure 2.6: Technologies involved for production of third and fourth generation of biofuel.

Source: Dutta et al. (2014).

The significant different of fourth generation biofuel from third generation was the use of technology such as genetic engineering, metabolic engineering and other biotechnology to optimized feedstock for higher yield of biofuel production. The use of algae as feedstock is because of its high photosynthesis and growth rate, however the production cost was high and facing problem with extensive downstream processing. From the figure it is obvious that the approaches was focus on modified and cultivate better algae feedstock that have more advantage such as high CO_2 capture rate, higher lipid production rate, and others. The processing procedure for third and fourth generation biofuel was similar, such as cultivation, microalgae, harvesting, extraction, conversion of biomass and others

2.5 Bioethanol

Bioethanol is ethanol produced by sustainable material, which is efficient, renewable, and less emission of greenhouse gases. Example of countries that able to produce bioethanol and major in world market was Brazil, United States of America, Canada and others. Bioethanol is one of the most promising automotive fuels as it can be easily produced from renewable resources, carbon neutral and widely used for transportation. The ethanol biofuel also the same types of alcohol found in alcoholic beverages, but ethanol itself cannot served as fuel and function as additive for gasoline. Example of ethanol fuel are E2, E5, E10, E15, E50, E85 and others. The "E" symbol indicate the percentage of ethanol was added to the gasoline. Bioethanol was one of the bioenergy that still have high possibility to improve and advancing. Those potential was highly depend on the source or production of renewable raw material such as agriculture residue, non-edible crops, and others, regarding any other issue such as optimization of technology and manufacture cost because the renewable and sustainable source raw material is core for sustainability of bioethanol production in future to substitute the fossil fuel that is going to deplete.

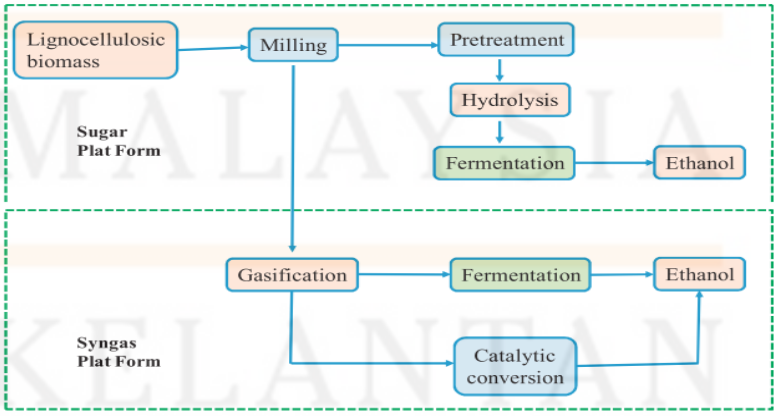

Figure 2.7: General pathway of lignocellulosic biomass to bioethanol conversion.

Source: Vohra et al. (2014).

2.6 Pretreatment of Lignocellulosic Biomass

2.6.1 Physical Pretreatment - Milling

Physical pretreatment doesn't involve any chemical substance. The objective for carry out physical pretreatment is to reduce the sample size and obvious contamination. The example of physical pretreatment is: mechanical extrusion, milling, microwave, ultrasound, pyrolysis, pulsed electric field, and others.

Milling is one of the physical method used to reducing the sample size of lignocellulosic biomass, it is one of the technique of mechanical grinding other than chipping, grinding and others. Particle size of biomass plays an important role on the overall sugar recovery. (Kumar & Sharma, 2017)Milling method can used to reduce the crystallinity of cellulose due to the shear forces generated during milling. Milling have many kind of way to carry out such as two-roll milling, hammer milling, colloid milling, and vibratory milling. (Kumar & Sharma, 2017) The milling method used in this research project is vibrating ball milling, it is found to be more effective in reducing cellulose crystallinity. (Kumar & Sharma, 2017)

2.6.2 Diluted and Concentrated Acid Pretreatment of Lignocellulosic Biomass

Acidic pretreatment use acid to breakdown outer structure of plant for higher yield of glucose release, acidic pretreatment able to achieve a good enzymatic digestibility of cellulose and remove hemicellulose or lignin from the lignocellulosic matrix. The general method for acid hydrolysis is: drying of biomass, submerged in acidic solution with specific temperature and pressure, after pretreatment is done separate the liquor from solid substrate, and neutralization before further step was carry out. The general principle is using acidic media to ensure the lignocellulose content is hydrolyse faster and release more glucose or other component that benefit fermentation process. Cellulose is the most significant component require to undergo degradation. The parameter of acidic hydrolysis are pretreatment duration, temperature, pH of solution, and others.

The general use of diluted acid concentration is about (0.2–2.5% v/v), it is mostly process with high temperature and pressure in order to speed up the reaction times in minute. (Bensah & Mensah, 2013). Reason for using such low concentration of acid was due to low production cost and ease of manufacturing. Also, using low concentration of acid during pretreatment able to release essential nutrient that enhance downstream processing. (Bensah & Mensah, 2013). The acid that used for acid pretreatment are mostly sulphuric acid, hydrochloric acid, and phosphoric acid, and others. Beside those mineral acid, the organic acid also have been found is able to performing dilute acid pre-treatment, the example of organic acid was: oxalic acid, maleic acid, and others. The most common mineral acid used for dilute acid pretreatment was sulphuric acid, although diluted nitric acid has higher glucose yield, but the byproduct from nitric acid pretreatment are difficult to remove by washing of pretreated substrates. (Bensah & Mensah, 2013). Although dilute acid pretreatment produce enhance the glucose yield release for fermentation, but

its disadvantage was less effective in removing lignin compare to other method such as alkaline pretreatment. (Bensah & Mensah, 2013) Also, regardless of the any % of acid concentration, it required corrosion-resistant bioreactor or fermenter, and this is contribute to increase of production cost; both diluted and concentrated acid pretreatment will burden the downstream processing because of required to remove the solid waste of neutralization (Bensah & Mensah, 2013).

The concentration of acid used which is more than 10% is considered as concentrated acid pretreatment. Differ than diluted acid pretreatment, concentrated acid hydrolysis is recommended to process in low temperature because the level of acidic enable the reaction to carry out faster than diluted acid method, and of course for safety purpose. Pretreated feedstock will left with rough molecular surface that enhance enzyme adsoption rate, in another word it can accelerate hydrolysis. Although concentrated acid pretreatment showed better performance than diluted acid, but it is limited in small scale or lab scale production. Large scale production using concentrated acid will causing corrosion issue on material and apparatus, increase costing in term of high cost of acid, required anti-corrosion equipment, and maintenance cost.

2.7 Simultaneous Scarification and Fermentation, SSF

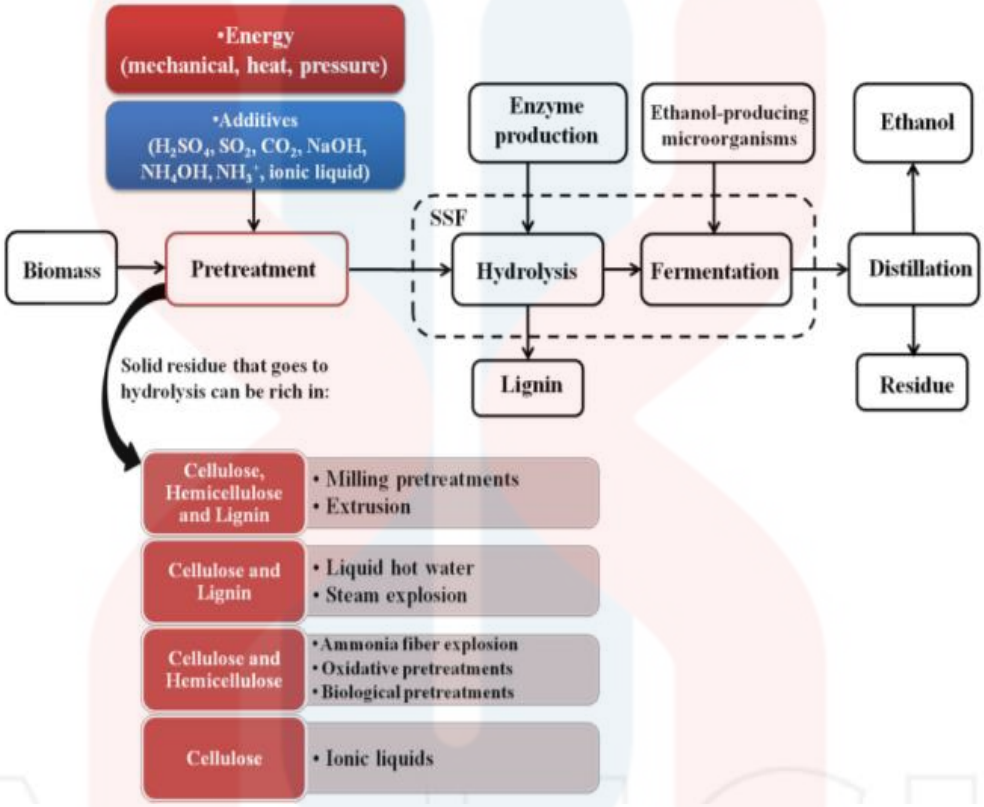

Figure 2.8: Complete pathway for ethanol production combined different pretreatment option with simultaneous scarification fermentation (SSF).

Source: Silva et al. (2013).

The SSF is process that that rapidly convert sugars into ethanol, it is effected by many other factor like types of enzyme, fermentative microbes, pre-treated biomass, and various process condition. During the fermentation process, the bacteria or yeast will use sugars as feed to carry anaerobic respiration, this process will produce ethanol and CO_2. In this research paper, the enzymatic reaction was combined with fermentation and this processed is called by Simultaneous Saccharification and fermentation (SSF). The enzymatic reaction hydrolyse cellulose to monomer and convert monomer to bioethanol.

The SSF method was considered as a better process than Separate Hydrolysis and Fermentation (SHF). (Dahnum, Tasum, Triwahyuni, Nurdin, & Abimanyu, 2015) From the view of capital investment, the combination of enzymatic reaction and fermentation able to reduce capital cost for industry because it reduce the number of unit procedure, equipment, facilities and man-power. Since it is showed more effective than fermentation process, several measurement has been studied to enhance the performance of SSF such as substrate loading, enzyme loading, microorganisms or yeast loading, temperature, and others.

2.8 Benedict Test for Glucose Content

Benedict test is using Benedict reagent for carry out qualitative or quantitative test of sample's glucose content. One litre of benedict's solution can be prepared from 100g of anhydrous sodium carbonate, 173g of sodium citrate, 17.3g of copper (ll) sulphate pentahydrate, and the rest is distilled water. Beside glucose, Benedict's test also identifies reducing sugar such as monosaccharide's and some disaccharides, which have free ketone or aldehyde functional group. When Benedict's solution is added to sample carbohydrate product and heat, the solution changes from green/blue to orange/red. The reducing properties of the carbohydrate will reduce the copper (ll) ions to copper (l) ions, which lead to the colour change. If the concentration of reducing sugar is high, the colour of benedict reagent will change closer to brick-red with greater precipitate amount because it is the colour of the copper (l) oxide. Beside copper (ll) sulphate, the sodium citrate and anhydrous sodium carbonate play an important role in benedict reagent: sodium carbonate provides the alkaline conditions which are required for the redox reaction; sodium citrate

complexes with the copper (ll) ions so that they do not deteriorate to copper (l) ions during storage. After acid pretreatment, if the starch or non-reducing sugar is present, it will not have reaction with benedict solution because of its non-reducing properties. Benedict's test for reducing sugars was used to determine the amount of sugars generated from the pretreatment techniques. The principle behind Benedict's test is that reducing sugars convert blue copper sulfate present in the Benedict's reagent to red copper oxide precipitate.

2.9 Freezing Test

The freezing point for ethanol is −114 °C, and freezing point for water is 0°C. After the recovery process, the ethanol water mixture was recovery from the fermentation broth, with other unknown impurity substance. The freezing point for ethanol water mixture was neither −114 °C nor 0°C, it will undergoes freezing point depression and the freezing point for mixture is differ by the concentration of ethanol in mixture. Freezing point depression is a phenomena that the decrease of the freezing point of a solvent, in this research which is water, on addition of non-volatile solute, like ethanol. (Echipare & Harju, 2018) Different concentration of ethanol-water mixture have its freezing point, at that certain freezing point the mixture with selected ethanol concentration of below it will freeze. When ethanol is present in the solvent, its freezing point is lowed from the original value of the pure water. In ethanol-water mixture, the ethanol will expelled from the solid and didn't solidified. This process is using the principle that ethanol have a much lower solubility in the solid phase of the water relative to the liquid state, and also called as fractional freezing (Chickos, Garin, & D'Souza, 2018). The similar way of fractional

freezing with ethanoic distillation is the use of different freezing point properties while the distillation is using properties of different boiling point, just like ethanol and water can be separate by fractional distillation. Fractional distillation work with any aqueous solution regardless of its miscibility. Unless the ethanol and water become azeotrope mixture, or else it can be separation by those fractional distillation process. The azeotropic state of water and ethanol is approximately at 95%, also called entectic point, if it reach azeotropic state the mixture is behave like single component that won't able to separate by thought fractional distillation.

2.10 High Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography is a chromatographic technique used to separate component in a mixture for identification and quantification of each and every component. Liquid chromatography is using normal phase and reverse phase as mode of HPLC. Reverse phase use non-polar component as stationary and polar solvent as mobile phase, for testing of ethanol the normal phase was used, which is using non-polar solvent as mobile phase and vice versa. The parameter need to be control is the type mobile phase, column, detector, wavelength, flow rate, injection volume, and other. The chromatogram is the result obtain after running HPLC. The principle for HPLC was: inject the testing sample into the column that have absorbance material using liquid solvent. The retention time in the chromatogram indicate the interaction of test subject with the column, if the retention time was fast it show the analyte flow out from the column very fast, and vice versa. It is able to study the concentration of the test subject in solution by study the peak area, peak height, and percentage of peak area.

There is two choice for the detector used for ethanol detection, which is absorbance detector and refractive index detector. The absorbance detector include ultraviolet detector (UV), visible light detector (VIS), and Photodiode Array detector (PDA). UV detector is consider the most common detector for HPLC analysis. The principle was: sample injected will absorb UV light, then the intensity of UV light that absorb by mobile phase will be observe and record to measuring the different of absorbance. VIS detector have longer wavelength detection that UV detector, but PDA can visualise the result in 3 dimension, meanwhile UV and VIS detector only available for 2 dimension. Refractive index used to test the change in reflex index of the sample, but not the absorbance. Its sensitivity is low if compare to UV detector , but it can detect all substance including sugar and inorganic ion that cannot be test using UV detector, also the selection of solvent is wider because RI detector can use solvent which has UV absorbance but UV detector cannot.

In this research project, the PDA detector was selected for the detection of ethanol. Suppose the RI detector should use for detection of ethanol because ethanol cannot using UV detector, due to the limitation of the instrumentation the PDA detector is selected to replace Refractive Index (RI) detector. But the principle of detection was indirect detection. The detection of a UV-Vis-transparent analyte is accomplished by adding light-absorbing ionic species into the mobile phase. The presence of the analyte is monitored by measuring a decrease in the light absorbed by the eluent as the analytes elute from the column. (Shen & Tomellini, 2007) The detectable component is continuously inject into the column with mobile phase, equilibrium is established for the detectable component between stationary phase and mobile phase as background level for detection. If the analyte is injected it will affect the distribution equilibrium, result in the positive or negative change in the detector response to produce peak in chromatogram.

2.11 Fourier Transform Infrared Spectroscopy (FTIR)

Fourier Transform infrared spectroscopy is a technique that using the principle of absorption or emission of a particle, either in solid, liquid, or gas to obtain the infrared spectrum. In simple word, the IR radiation is release to the sample, some radiation will pass through and some will release. The fundamental of FTIR is using the absorbance of light in the infra-red region in electromagnetic spectrum for all test subject, the absorption is specifically represent the bond in the molecules. (Birkner & Wang Qian, 2015) FTIR using a beam that contain of many frequencies of light at once, then detector the amount beam is able to absorb by the sample. The selection of light beam is more advance than previous method, which is using monochromatic light beam, a beam that composed of one specific wavelength to the sample, and repeat it using other beam with different wavelength.

When beam splitter receive the light from the source, it will splits the light into two beams that passed to the movable mirror to fixed mirror, then reflected from those mirror back to beamsplitter. At the sample time, some fraction of light from the source passes to the sample, now all the light is focused on the sample then refocused to the detector (Bradley, 2018).

The combine signal is called as interferogam, it is obtained by varying the retardation and recording the signal from the detector for various values of the retardation, also it is built by measurement of difference positions signal from the moving mirror, then convert by Fourier transform to the infrared spectrum.

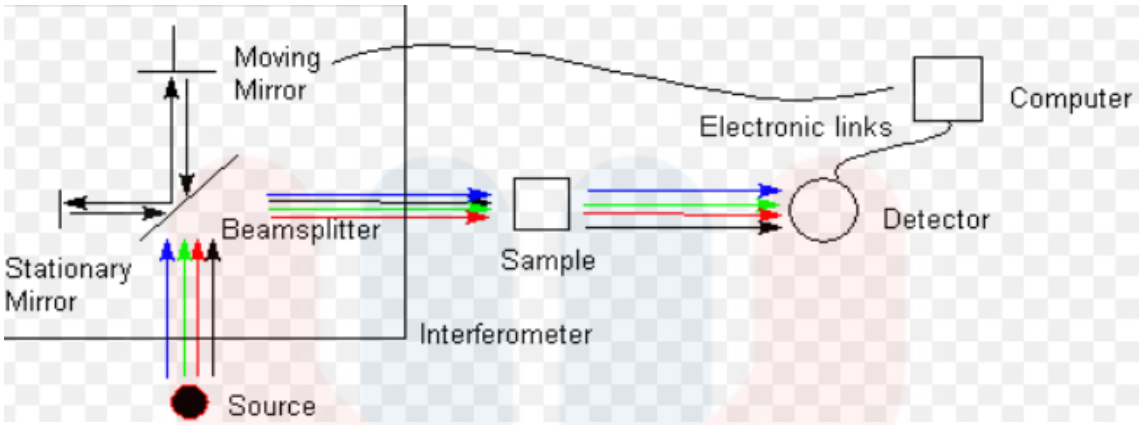

Figure 2.9: The mechanisms of the Michelson interferometer in the FTIR.

Source: Gable. (2013).

Assuming the covalent bond is spring in equilibrium, when applied infrared radiation it will stretched and compressed, causing the covalent bond vibrate in stretching, scissoring, and other mode. The most useful bands in an infrared spectrum correspond to stretching frequencies. When a chemical sample is exposed to the action of IR light, it can absorb some frequencies and transmit the rest. Some of the light can also be reflected back to the source. From all the frequencies it receives, the chemical sample can absorb specific frequencies and allow the rest to pass through it. The detector detects the transmitted frequencies, and by doing also reveals the values of the absorbed frequencies.

There is three types of IR bands, which is strong, medium, and weak band that different in relative intensities in the infrared spectrum. The stronger IR band, the larger the area of y-axis cover by this band. (Sergio, 2003) There is also some information we can obtained through observation of the broad or narrow of the band shapes, the triple bond molecules showed the narrowest band while the single bond molecules showed the widest band, such as O-H bonds.

IR spectral able to show the presence or absence of specific functional groups, it can present the fingerprint that is specific for each molecules, however it didn't provide detailed information of molecular formula or structure, but only functional groups or molecular fragment. In order to get more detailed information, FTIR required to conjugate with other characterisation method and technique.

The spectrum in figure 4.6 showed in this analysis was in transmission mode, as the most common representation, showed transmission intensities as y-axis and frequency of wavenumber in x-axis. The IR bands that fall nearest to zero % intensities and cover almost all of the y-axis showed strong IR Bands, while the IR bands fall the less is the weakest bands. Also, from observation of the bands shapes, different functional group showed diffent band size, which is narrow or broad. There is 2 way to study the IR spectrum, left side of the spectrum within range to 1400 to 4000 cm^{-1} is the most easy area to study, here showed the stretching band of the molecules in your molecules that is easier to recognize and analysis. The right side of the spectrum within range 600 to 1400 cm^{-1} is the fingerprint region that showed the bending of the bond, which is more complex and overlapping than left region, but it is informative to identify the compound.

2.12 Silver Mirror Test

Silver mirror test, also named as Tollens' test to distinguish aldehyde and ketone. The present of carbonyl group which is the functional group consist of carbon – oxygen double bond, can further identify the present of aldehyde and ketone. Aldehyde consist of carbonyl center, a carbon double-bonded to oxygen, also bonded to hydrogen and an R group; the ketone group contain a carbonyl group bonded an R group and R' group.

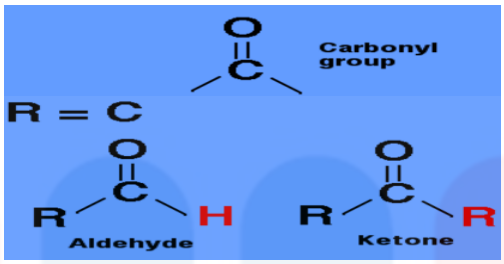

Figure 2.10: Different between aldehyde and ketone in same carbonyl group. Source: E.Ophardt. (2003).

The tollens' reagent is used in silver mirror test, and it is prepared by silver nitrate, sodium hydroxide, ammonium hydroxide, and others. The Tollens' reagent need to be fresh prepared because it cannot be stored as silver nitride can be formed from the solution if store for too long, which has risk to cause explosive. Aldehyde is known as carbonyl compounds which showed a carbon to oxygen double bond with a hydrogen attached to it. There is 2 reaction in preparation of Tollens reagent, first is the formed of silver oxide, Ag_2O, which is the brown solid precipitate; the second reaction is dissolving of silver oxide using aqueous ammonia to form diamminesilver (I) complex ($[Ag(NH_3)_2]^+$).

$$2\ AgNO_3 + 2\ NaOH \rightarrow Ag_2O\ (s) + 2\ NaNO_3 + H_2O \qquad (2.1)$$

$$Ag_2O\ (s) + 4\ NH_3 + 2\ NaNO_3 + H_2O \rightarrow 2\ [Ag\ (NH_3)_2]NO_3 + 2\ NaOH \qquad (2.2)$$

If the test subject is present with aldehyde, the $[\boldsymbol{Ag(NH3)2}]^+$will act as oxidizing agent and oxidizes the aldehyde to a carboxylate ion, at the same time the $[\boldsymbol{Ag(NH3)2}]^+$ is reduced to silver element and ammonia. The element silver will form a silver coating in the inner of the vessel used, causing the "silver mirror" to produce.

$$2\,[Ag(NH_3)_2]^+ + RCHO + H_2O \rightarrow 2\,Ag(s) + 4\,NH_3 + RCO_2H + 2\,H^+ \quad (2.3)$$

In this research work, the silver mirror test is used to identify the present of aldehyde in sample, which is the inhibitory substance for ethanoic fermentation.

CHAPTER 3

METHODS AND MATERIAL

3.1 Material and Apparatus

The chemical used in this study were potassium dihydrogen phosphate (KH_2PO_4), peptone, yeast extract, sulphuric acid (H_2SO_4), sodium hydroxide (NaOH), silver nitrate ($AgNO_3$), ammonia (NH_3), and distilled water. *Panicum maximum sp.* (guinea grass) was taken freshly within the area in UMK while the ragi tempeh was purchased from the Jeli Town market.

Apparatus that used in this study was test tube, falcon tube, inoculating loop, Bunsen burner, petri dish, parafilm, beaker, media bottle, schott bottle, pH meter, aluminium foil, spatula, measuring cylinder, reagent bottle, forceps, rotary evaporator, vacuum pump, buchnel funnel, hot plate stirrer, filter paper, poly bag, syringe filter, gloves, filter funnel, glass rod stirrer, pestle mortar and pipette.

3.2 Equipment

To carry out this study, certain instrument were selected such as Fourier Transform Infrared Spectroscopy (FTIR) (Thermo $Scientific^{TM}$ Nicolet IZ10), Bioreactor ($BIOSTAT^{®}$ Aplus), High Performance Liquid Chromatography (HPLC) (HPLC –UV-DAD Shimadzu Prominence), Centrifuge.

3.3 Method

3.3.1 Identification of the Amount of Bacteria in Ragi Tempeh

The bacteria source used from this mini project was came from ragi tempeh. The ragi tempeh is brought from Jeli Town market. The serial dilution and simple dilution was carry out to identify the bacteria amount and morphology of the bacteria. For carry out serial dilution, 1g of sample were ground to powder and poured into media bottle that filled with 99ml distilled water to fill up to 100ml. The dilution concentration was anged from10^{-2} to 10^{-5}. The culture media contained 7.5 g/L peptone, 5g/L yeast xtract. 1 g/L KH_2PO_4 , and agar powder. The medium is autoclaved at 121°C for 15 min. After dilution, using inoculating loop the streak the different diluted solution on the medium and incubated at 37°C for 2-3 days.

3.3.2 Preparation of *Panicum maximum* Bagasse

The grass *Panicum Maximum* was collected around area of University Malaysia Kelantan Campus Jeli. Sample was wash and cut into small pieces. Next, the bagasse was dried under sunlight for 2-3 days to remove water content. After dried, the bagasse was mill using ball-mill machine into powder form, it is collect and store in poly-bag.

3.3.3 Acid-Pretreatment of *Panicum Maximum* Bagasse.

The bagasse at a solid loading of 10% (w/v) was mixed with diluted sulphuric acid at different concentration of 2.5%, 5%, 10%, and 15% (v/v). The sulphuric acid with different concentration prepared by mixing concentrated sulphuric acid (98% - 100%) with distilled water. The mixture is then proceed to pre-treatment process by autoclave at 121℃ for 15 min. After pre-treatment, remove the solid bagasse of acid solution by vacuum filtration and collect the filtrate. Noted that the pH of the solution is extremely low due to the high concentration of sulphuric acid was use. The titration method is used to titrate 2M sodium hydroxide solution to neutralize the pH of the solution to suitable pH. After neutralization, remove the suspended substance in solution by vacuum filtration again. Before further proceed of experiment, use benedict solution to test the glucose content in pre-treated solution.

3.3.4 Fermentation using Bioreactor and Benchtop Shaker

The model of the bioreactor is BIOSTAT® A entry-level bioreactor/fermenter. 50g of ragi tempeh powder, 200 – 250 ml of acid pre-treated solution, and 800ml of nutrient media was added into the bioreactor. The nutrient media was made up by 7.5 g/L peptone, 5g/L yeast extract. 1 g/L KH_2PO_4 and distilled water to make up to 800ml. Beside Those main ingredient for fermentation, the choice for acid and base used to control the pH of bioreactor was 250ml of 1M sulphuric acid and 250ml of 2M sodium hydroxide. Since the fermentation process belongs to anaerobic respiration the bioreactor should be seal and no air allow to enter, also it is no need to use antifoam for the fermentation. The RPM of the impeller is set to 100, and the dissolved oxygen amount in the broth should control to range 0.1 to 1 and shouldn't be too high. The temperature was set at 35°C and control by the electrical heating jacket. Duration for fermentation was 2-3 days. The different pH used as parameter for fermentation was 5, 6, 7, and 8. Before and after use of bioreactor it should be autoclave to ensure no contamination would affect the fermentation process. Another fermentation process was carried out using benchtop shaker

3.3.5 Recovery of Fermentation Broth by Centrifugation and Rotary Evaporation

After fermentation was done, the fermentation broth was collected and proceed to centrifugation and rotary evaporation by rotary evaporator. The fermentation broth was equally divided filled in all centrifuge bottle and centrifuge using high capacity centrifuge. The RPM and duration was set to 1000 and 20 minute. After centrifugation and remove

solid content, the fermentation broth was proceed to rotary evaporation. 200ml of fermentation broth was placed in 500ml round bottom flask, too high amount of fermentation broth filled have risk to spilled out when boiling, effect the amount of ethanol collect. The rpm of the rotor was 100, and the temperature of the water bath was set to 76°C. The collected sample in collecting flask in store and proceed to HPLC testing.

Sample collected from bioreactor

No	pH	Instrument used
Sample 1	5	bioreactor
Sample 2	7	bioreactor
Sample 3	5	shaker
Sample 4	6	Shaker
Sample 5	8	shaker

3.4 Characterisation

3.4.1 Freezing Test

After rotary evaporation, the samples was labelled and stored in the freezer. The temperature of the freezer is set to -20°C. Observe the change of the sample after 24 hours to check whether it is freeze to solid or not.

3.4.2 Silver Mirror Test

Chemical used in silver mirror test is 0.3M silver nitrate solution, 0.3M sodium hydroxide, and aqueous ammonia solution. Pour 2 mL of silver nitrate into a clean test tube, drop 2-3 drops of sodium hydroxide in the test tube too, the brownish precipitate will formed. After that, use dropper to drop aqueous ammonia into the test tube until the brownish precipitate disappear and the solution become colourless and clear, the fresh Tollens' reagent is prepared and ready to use. 5ml of sample is added into the mixture, the test tube is shake well and placed in the hot water bath until the silver mirror is formed.

3.4.3 Fourier Transform Infrared spectroscopy (FTIR)

Before using FTIR machine, use 70% ethanol to clean the sample compartment surface of FTIR machine. Use dropper to drop 1-2 drop of sample on the compartment surface and waited for the IR spectrum showed up in computer.

3.4.4 High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography machine was used to do qualitative and quantitative test. 1.5ml of the sample was injected into HPLC system to determine the bioethanol yield. The recommended analytical conditions was:

- LC column: C_{18} column
- Mobile phase: 0.1% formic acid solution
- Flow rate: 1ml/min
- Injection volume: 20uL
- Detector: 254nm PDA detector
- Calibration method: comparison with ethanol standard solution

CHAPTER 4

RESULT AND DISCUSSION

4.1 Benedict Test for Glucose Content after Pretreatment

The purpose for carry out glucose assay it to test the amount of sugar release after pretreatment, also it was able to confirm the presence of sugar prior for fermentation. This benedict test result gives semi-quantitative estimation amount of the reducing sugar in the sample, it is called semi-quantitative because using colour as data only can give rough estimation, not detail quantitative result that can show in statistic or number. The colour change of benedict reagent was showed in table 4.1 below:

Table 4.1: standard result of benedict test

Colour	Approximate amount of sugar
Blue	No sugar
Green	0.5%
Yellow	1.0%
Orange	1.5%
Red	2.0%
Brown	Highest level of sugar

Source: Danmaliki, Muhammad, Shamsuddeen, & Usman. (2016).

Table 4.2 recorded the colour change for all 6 test subject. The concentration of diluted sulphuric acid concentration is 0.6%, 0.9%, 1.2%, 1.5% and 5%. Result in figure 4.1 showed that the colour was appear as green for 5% sulphuric acid concentration, and dark green colour for 0.6%, 0.9%, 1.2% and 1.5% sulphuric acid concentration. Meanwhile, from figure 4.2 the Benedict's test for 15% sulphuric acid concentration showed yellow colour. All of six sample was carry out acidic hydrolysis in same condition, which is autoclave at 121°C for 15 minutes, all acidic solution has 10% (v/w) solid loading of bagasse. Acidic pretreatment using 15% sulphuric acid showed the best result among others, yellow colour represent approximately 1.0% amount of glucose after pretreatment, while other sample having acid concentration of lower or equal to 5% show green colour that represent approximately 0.5% amount of glucose. 15% sulphuric acid concentration now selected as acid concentration with best performance and used it on following procedure. Although 15% sulphuric acid not belongs to diluted acid pretreatment, but it is acceptable to apply in further procedure because there is no use of hydrolysis enzyme, such as cellulase before fermentation. Concentrated acid pretreatment able to release higher glucose yield compare to diluted acid pretreatment, which can cover the inadequate of not using in enzyme during fermentation.

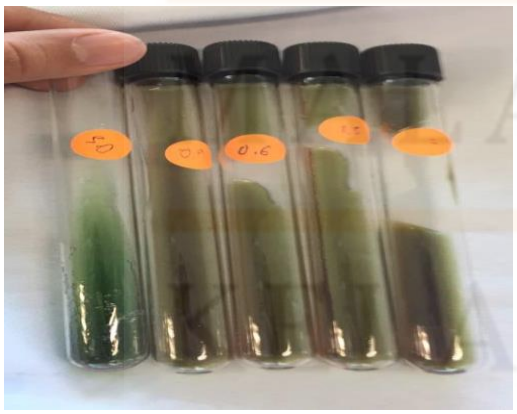

Figure 4.1: Benedict test for diluted acid pretreated sample.

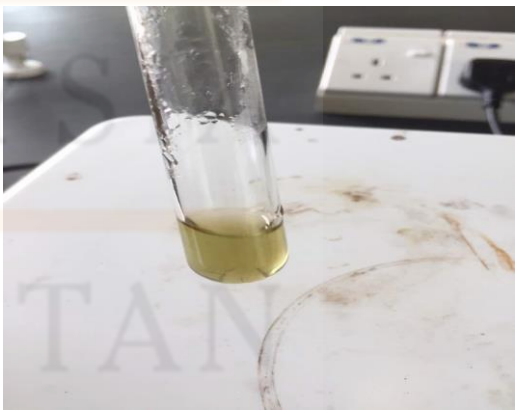

Figure 4.2: Benedict test for concentrated acid pretreated sample.

Table 4.2: Result of benedict test for 6 acid pretreated sample.

Percentage of sulphuric acid used	Colour change after benedict test
0.6	Dark green
0.9	Dark green
1.2	Dark green
1.5	Dark green
5.0	Light green
15.0	Pale yellow

4.2 Freezing test

The sample obtained from rotary evaporation is stored in falcon tube and freeze it in the -20°C freezer. The observation of freezing test has been done and tabulated in Table 4.3 and 4.4. After 48 hours of freezing, all the sample was freeze to solid. From direct observation, there is no liquid content appear in the sample. There is two possibility outcome to explain this: no ethanol content can be found, or the concentration of ethanol is extremely low. Although all of the sample has frozen and become solid, but there is still possibility that it is because of the concentration of ethanol in mixture is too low, maybe even lower than 1%. Also, it is assume that the ethanol content might be remain liquid in the middle of the frozen solid that cannot be observe directly. Further analysis of the mixture by HPLC will be proceed to determine the present of ethanol in sample. The following figure showed the freezing point for different mixture of water and ethanol.

Table 4.3: Observation of physical state of ethanol produced using benchtop shaker at -20 °C freezing point.

Time	Physical state of ethanol mixture at -20°C		
	pH 5	pH 6	pH 8
24 hours	Solid	solid	solid
48 hours	solid	solid	solid

Table 4.4: Observation of physical state of ethanol produced using bioreactor at -20 °C freezing point.

Time	Physical state of ethanol mixture at -20 °C	
	pH 5	pH 7
24 hours	solid	solid
48 hours	solid	solid

Figure 4.3 showed the freezing point of sample with different ethanol concentration. When the temperature is -20 °C then the only the mixture that have ethanol concentration less than or equal to 30 % will freeze. It is assume that the concentration of ethanol is too low, cannot be identify by using freezing test.

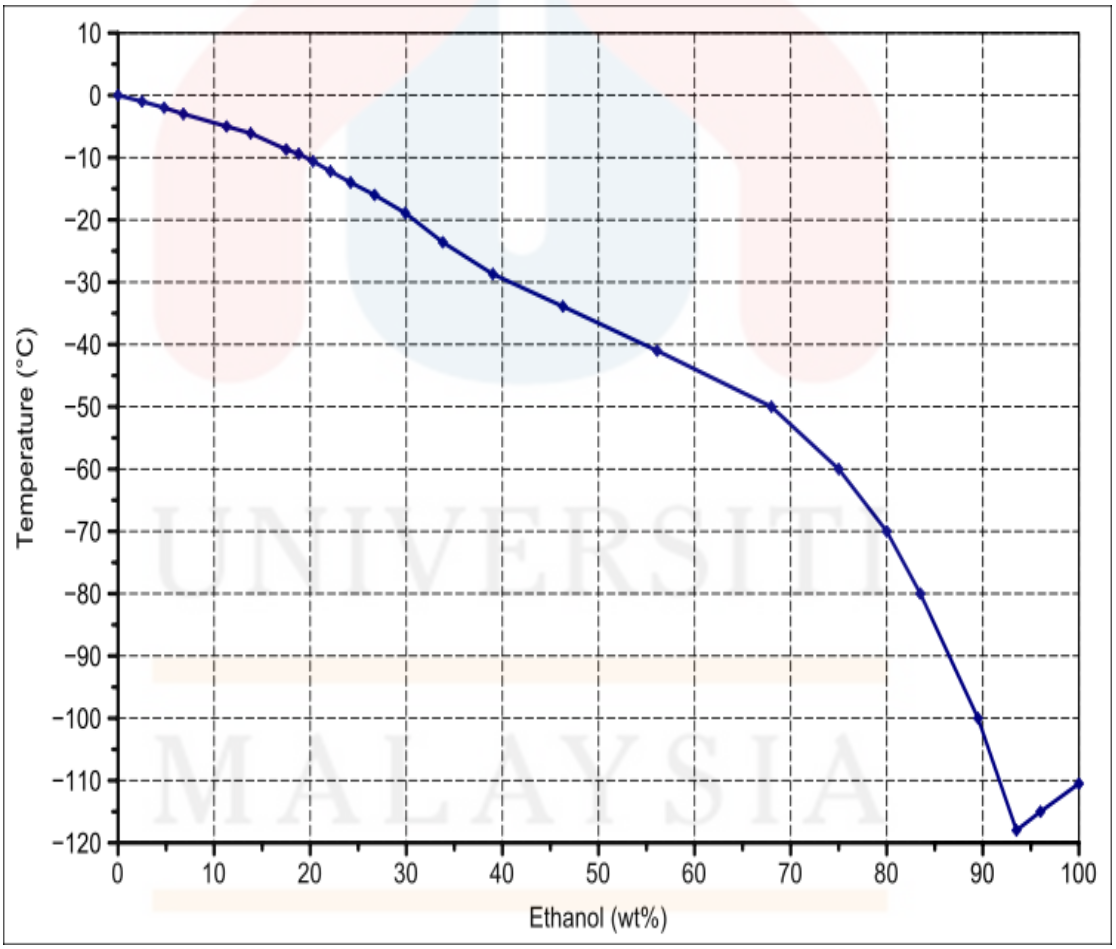

Figure 4.3: The standard graph of relationship of different ethanol concentration with its freezing point.

Source: Dean. (2008).

4.3 Tollens test

After running the Tollens' test, the figure 4.4 showed the result for all 5 sample. Counting from the left, the first test tube showed grey-cloudy solution with presence of black precipitate after remove the liquid content; unfortunately the second test tube is accidentally broken, but from the figure the black precipitate is formed in the inner of the test tube and attach on surface. The first & second test tube is the sample carry out fermentation using bioreactor with different pH, the pH of first test tube is 5 while the second test tube is 7. Although no silver mirror but only cloudy solution with some black precipitate present, but it is still positive result for Tollens test, means the aldehyde is present in this 2 sample. The third to fifth test tube contain the sample produced by using shaker with different pH as parameter, the pH for sample 3, 4, 5 is 5, 6, 8. The sample 4 is showed the best performance of silver mirror test, the silver element is form in inner test tube surface. The sample 3 showed same result as sample 1, which is grey-cloudy solution with some black precipitate present. The sample 5 showed brown colour solution after test, it is consider negative result because it is neither form silver mirror nor black precipitate with dark-cloudy solution appear. This Tollens test showed sample 1 to 4 present aldehyde that might causing the inhibitory effect of ethanoic fermentation and reduce the ethanol yield.

Sample 1 Sample 2 Sample 3 Sample 4 Sample 5

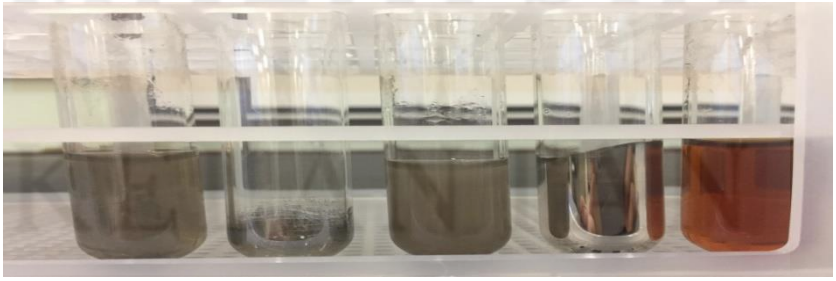

Figure 4.4: Result of the Tollens's test for all 5 sample.

Figure 4.5 showed all the possibility molecules can be produce by using acid pretreatment of lignocellulosic feedstock. Using acid pretreatment method will produce inhibitory component. (Kumar & Sharma, 2017) The example of inhibitory substance that might bring inhibitory effect to ethanol production was furans, aliphatic carboxylic acids, uronic acids, and phenylic compounds. (Jönsson & Martín, 2016) The hydrolysis of acetyl groups and esterified phenols in hemicellulose by acid pretreatment produce aliphatic carboxylic acids such as acetic acid, formic acid, levulinic acid, and phenylic compounds such as phenolic compounds, and non-phenolic aromatic compounds. (Jönsson & Martín, 2016) other than furan aldehyde and phenylic aldehyde, there is also present of small aliphatic aldehyde from acid pretreatment. (Jönsson & Martín, 2016) Those inhibitory component will cause the inhibition of microorganisms, which causing limited growth of fermentative microorganisms and lower ethanol yield. The result of the silver mirror test showed the present of aldehyde in all example, except sample 5. The present of aldehyde is suspected to be the main reason for low ethanol yield after fermentation in this study.

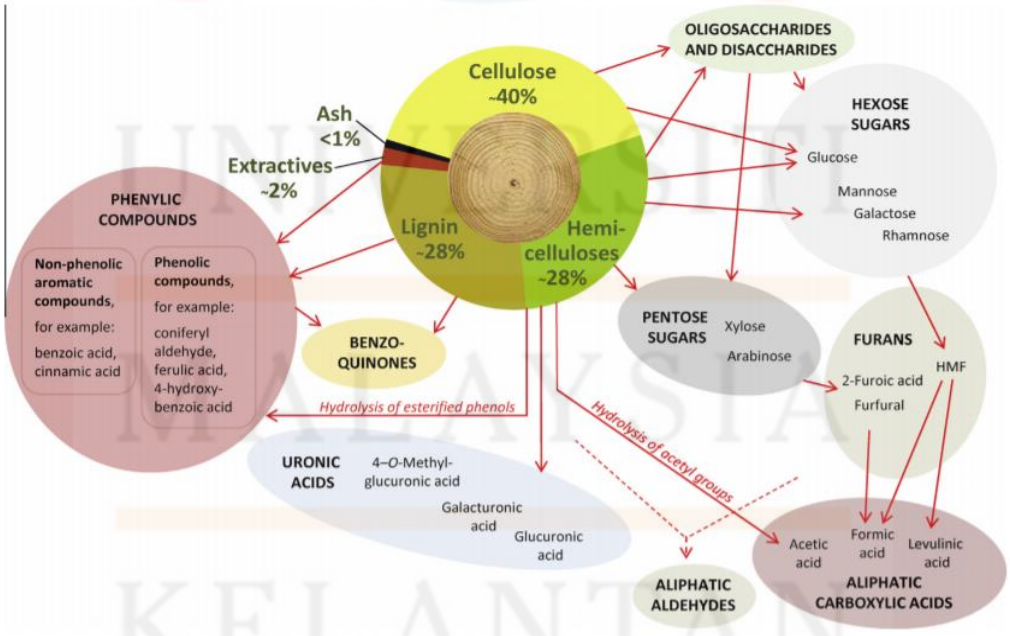

Figure 4.5: Degradation products from lignocellulose as result of pretreatment under acid conditions.

Source: Jönsson & Martín. (2016).

4.4 FTIR Spectroscopic Analysis

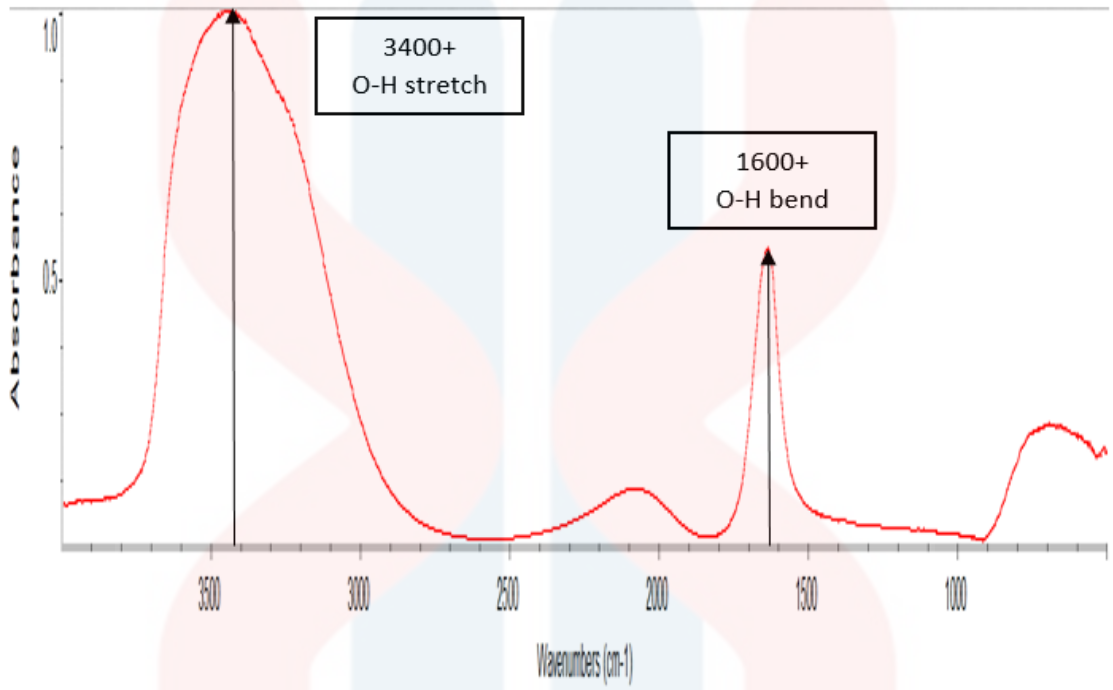

Figure 4.6: FTIR spectra of water in absorbance mode.

The FTIR software used to analyse the sample is OMNIC™. The figure 4.7 showed the spectrum diagram taken from OMNIC™ library. 70% ethanol and distilled water was choose to be the reference solution to compare with the sample that obtain after recovery from the fermentation broth, which is suppose to be ethanol-water mixture. The water was used as negative control for this study while the 70% ethanol is used as positive control. The 70% ethanol solution have 71.13% matched with the standard ethanol spectral in database in HR Aldrich Solvent library. The spectral of water was taken from the HR Hummel Polymer and Additives database, that is more accurate than testing distilled water on FTIR again. Spectra of distilled water showed the presence of a broad spectra at approximate 3400 cm^{-1} is O-H stretch, and a comparatively narrower spectrum at approximate 1600 cm^{-1} as O-H bend.

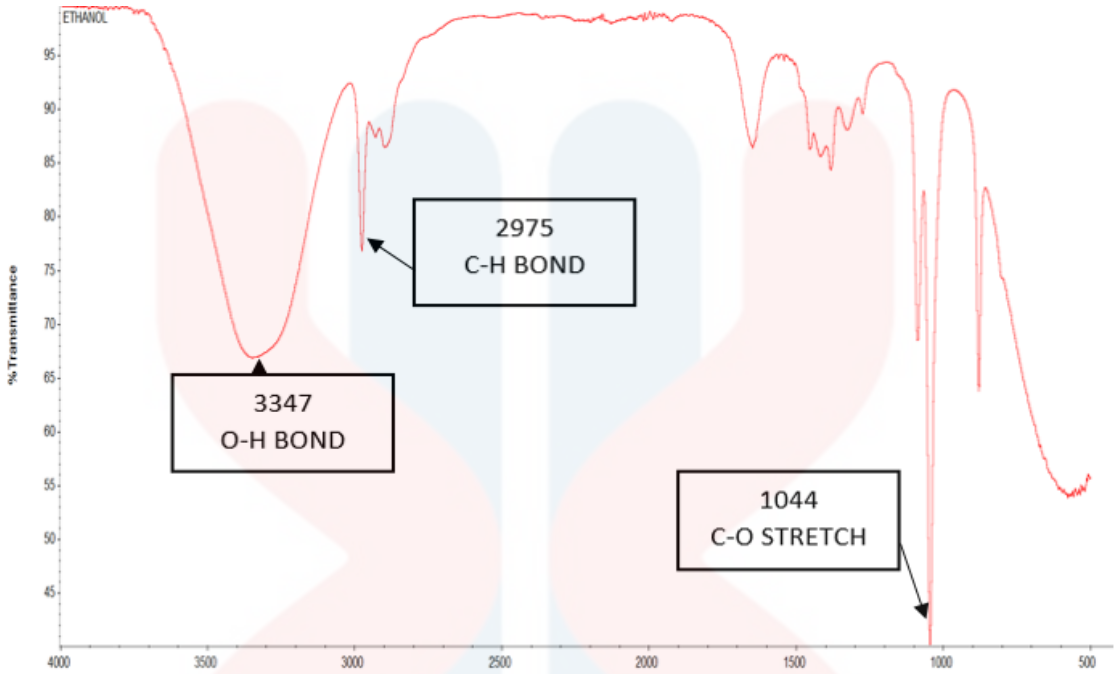

Figure 4.7: FTIR spectra of 70% ethanol in transmission mode.

Table 4.5 showed the standard functional group and fingerprint region for ethanol in infrared spectrum with the support of reference. Theoritically, the peak used to indicated ethanol in FTIR spectrum is O-H bond (3000 – 3600) cm^{-1}, C-H stretch (2800 – 3000) cm^{-1}, and C-O (1020 – 1200) cm^{-1} bond in fingerprint region. The FTIR spectra of the sample that fermentation using both bioreactor and shaker is are showed in figure 4.7 and figure 4.8. It only showed the peak of O-H stretch at range within 3312 – 3319 cm^{-1} and another peak of O-H bend ar range 1600 – 1636 cm^{-1}. Comparing all 5 sample with the positive and negative control, the functional group of all sample showed high similarity with the functional group of negative control, which is water. There is no peak of C-H stretch in left region of the spectra and C-O stretch in fingerprint region, which is the most significant prove for present of ethanol. The table 4.6 showed that only water is able to detect by this FTIR test. However, there is still posibiltiy that the

concentration of ethanol is too low that cannot be detected by FTIR. There is limitation of FTIR spectrometer to detect ethanol, the concentration of 0.08 % v/v ethanol-water mixture and below is unable to detect by FTIR spectrometer , (Boyd & Kirkwood, 2008) it still have possibiltiy that the ethanol is present in the sample, but its concentration is too low that cannot be detect by FTIR. Perhaps HPLC analysis can further prove the presence of ethanol.

Table 4.5: The standard absorption of functional group and fingerprint region of ethanol

Reference	Functional Group		Fingerprint
	O-H (cm^{-1})	C-H (cm^{-1})	C-O (cm^{-1})
(Adina Elena Segneanu, Ioan Gozescu, Anamaria Dabici, & Paula Sfirloaga and Zoltan Szabadai, 2012)	3000 – 3600	2800 – 3000	1020 – 1120
(Corsetti, Zehentbauer, McGloin, & Kiefer, 2015)	3300 – 3600	2850 – 3000	1050 – 1200

Table 4.6: The collected data of each significant peak present in analytes, and both positive and negative control.

Test Sample	Functional Group			Fingerprint
	O-H stretch (cm^{-1})	O-H bend (cm^{-1})	C-H stretch (cm^{-1})	C-O (cm^{-1})
70% ethanol	3347	-	2975	1044
pH 5 bioreactor sample	3312	1636	-	-
pH 7 bioreactor sample	3312	1636	-	-
pH 5 shaker sample	3319	1636	-	-
pH 6 shaker sample	3313	1636	-	-
pH 8 shaker sample	3319	1636	-	-
Water	3400	1600	-	-

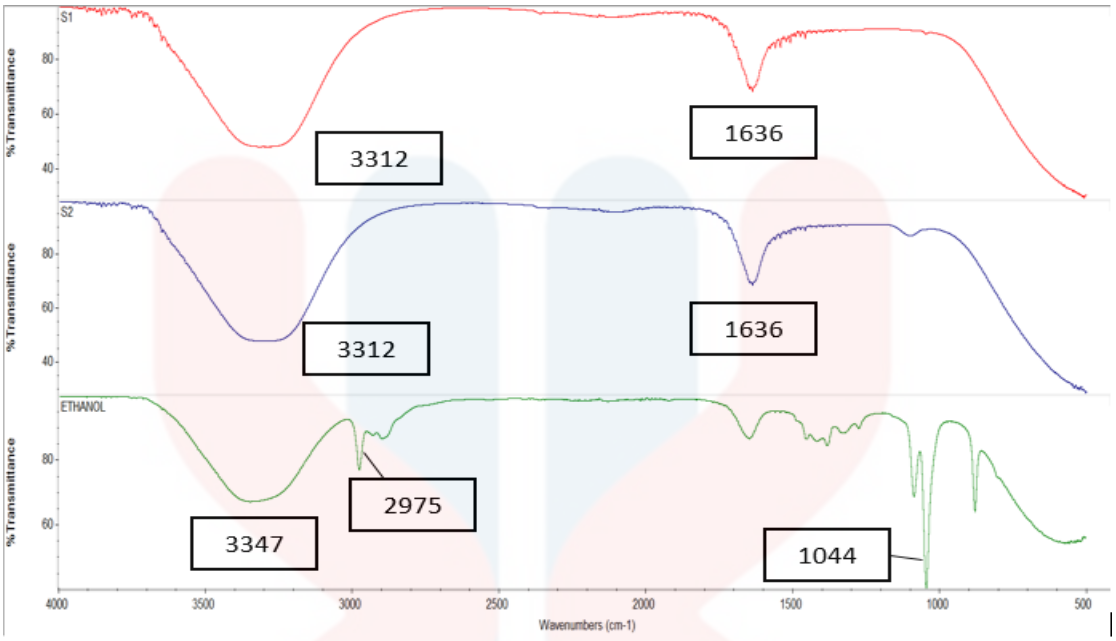

Figure 4.8: combined FTIR spectra for sample produced using bioreactor with fermentation pH5 (S1), fermentation pH 7 (S2), and 70% ethanol.

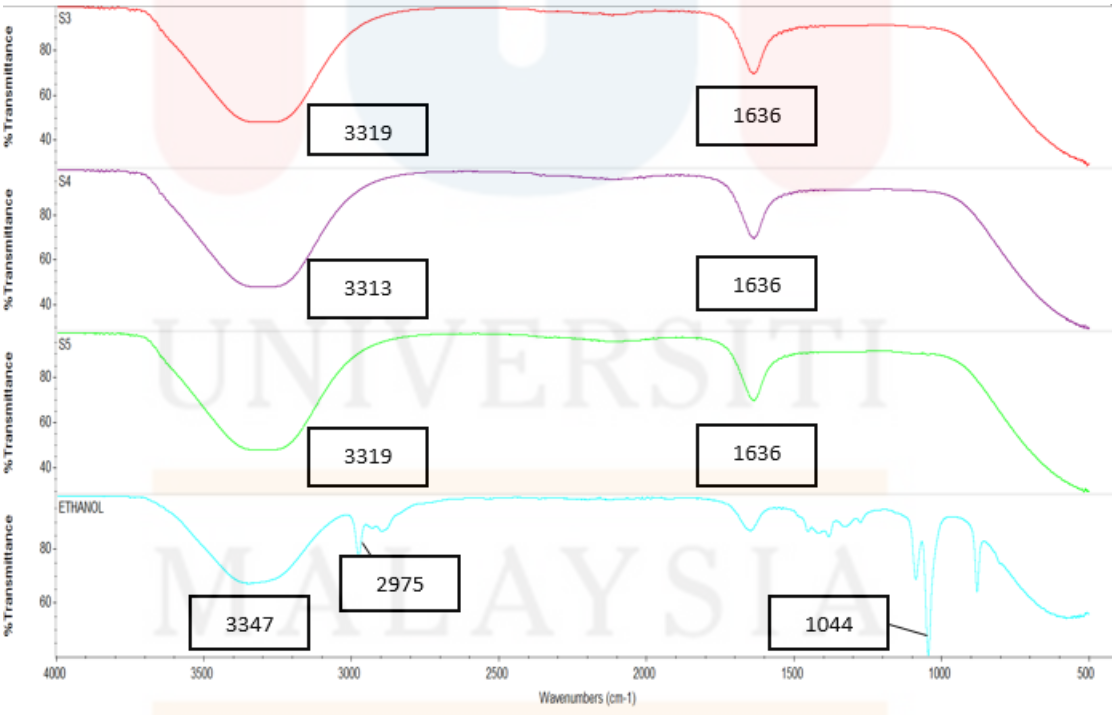

Figure 4.9: combined FTIR spectra for sample produced using shaker with fermentation pH5 (S3), fermentation pH 7 (S4), fermentation pH 8 (S5) and 70% ethanol.

4.5 HPLC analysis

Figure 4.10 is the calibration curve that showed the peak are for different concentration of ethanol. The concentration of ethanol use to construct this graph was 25%, 50%, 75%, and 100%, using absolute ethanol and distilled water by v/v. The retention time for these 4 concentration is 3.357 min, 3.382 min, 3.358 min, and 3.351 min, the acceptable range of the retention time of ethanol is within 3.2 – 3.4 min. Its average producing peak area that increase directly proportional with concentration of ethanol. R^2 of the graph showed 0.9902, the value that excess 0.95 means the data are fitted regression line. This calibration curve will used to find the present or absent of ethanol in sample, also the concentration of ethanol in sample if it is present.

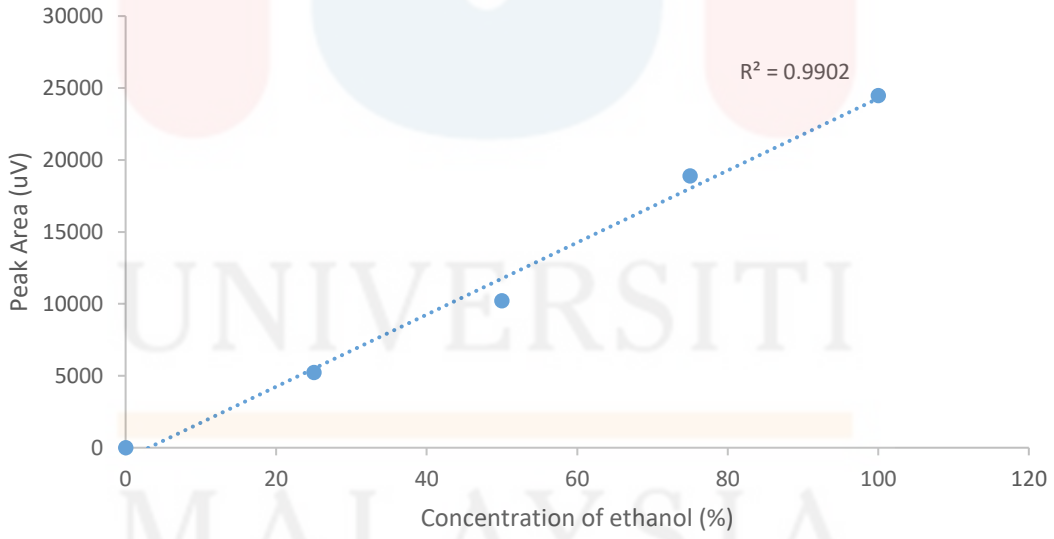

Figure 4.10: The standard curve for 0 - 100% ethanol concentration using HPLC.

Area of the peak and height of the peak has 2 different concept, figure 4.11 and figure 4.12 summarise the useful information of ethanol concentration in sample from figure 4.13 to figure 4.18. The area of the peak refer to the concentration of the particular compound. The highest peak area was obtained from figure 4.13, which showed almost 30000Uv of height and it is significant difference among other peak in all chromatogram, but it didn't mean the concentration of ethanol in sample 1 is high. Since the baseline drifting problem is very serious, studying the peak area percent showed in figure 4.12 will more accurate compare to figure 4.11. The figure 4.12 showed that except sample 3 all other sample only tested approximate 1% of ethanol present in the sample. The abnormal percentage of peak area is due to the baseline drifting problem too.

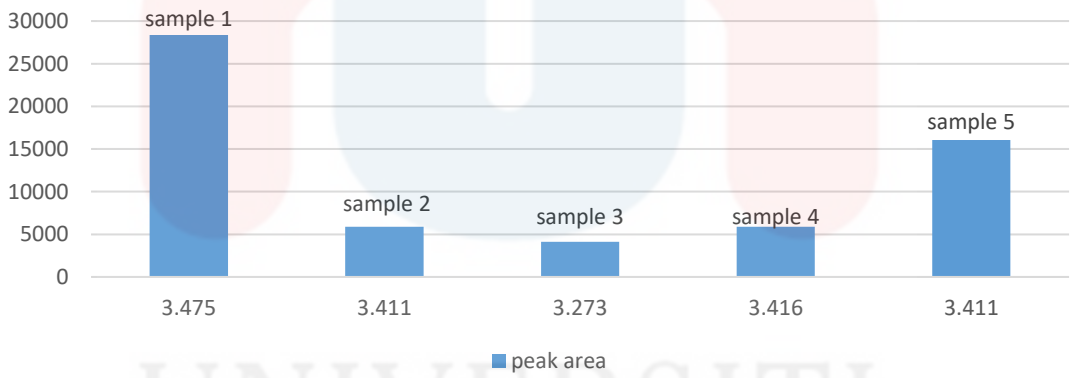

Figure 4.11: The peak area of the ethanol present in chromatogram of sample 1 to 5.

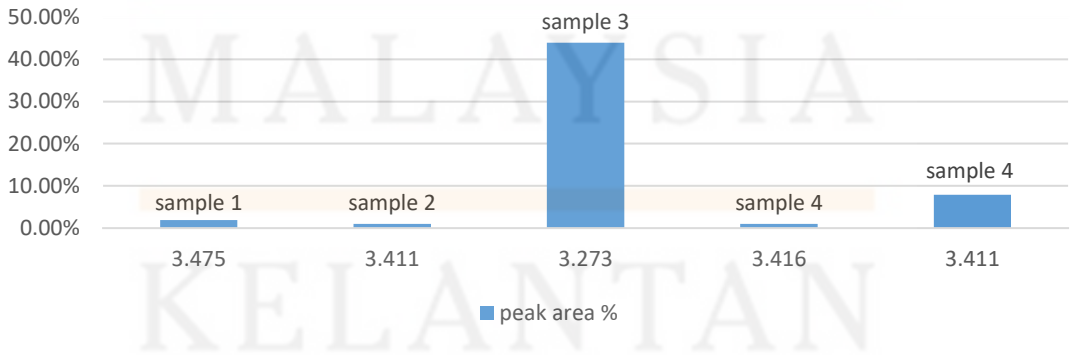

Figure 4.12: The percentage of ethanol peak area in chromatogram of sample 1 to 5.

4.5.1 Troubleshoot of HPLC chromatogram

From the observation of figure 4.13 (a) to (e), all of the chromatograms showed baseline shift problem, both positive and negative shift. Baseline drift might cause by column temperature fluctuation, even a little change of column temperature will cause the baseline rise and fall. This fluctuation might affect UV detectors at high sensitivity or indirect photometric mode. Also, the drifting problem might come from mobile phase, such as mobile phase contamination, mobile-phase mixing problem, and change of the flow rate. Since the detector used is UV detector, there is possibility that the absorbance didn't set at maximum. Among all the chromatogram, figure 4.13 (e) considered as normal chromatogram compare to other chromatogram, but the problem appear is rounded peaks and broad peaks. Again, there might causing by error of detector or mobile phase. Overall, the problem in the column is the source for those abnormal phenomena in chromatogram. It is strongly recommended to filter the mobile phase and sample before inject into HPLC, and the use of guard columns able to prevent particles and strongly retained compound to accumulated in column that will affect the absorbance reading from detector. Also, the probable cause for negative peaks at figure 4.13 (b) and figure 4.13 (d) is the mobile phase have more absorptive than sample components to UV wavelength. Since the indirect method is used, it is suggest to change the polarity of the mobile phase.

From figure 4.13 (a) to (e), the data showed the present of peak within the retention time range of 3.2 min to 3.4 min. Compare to standard curve of ethanol, this range of retention time indicate the present of ethanol. Figure 4.13 (f) showed the chromatogram of distilled water. Although this is not deionised water but is distilled water, but supposed the chromatogram show flatted baseline. The HPLC grade water belongs to

ultra-pure water with very low UV absorbance, it should filtered through 0.2 micrometre filter. At least, the water used for HPLC should carry out both distilled and deionized process. Present of impurities will raise the UV absorbance of water that will affect the analyte determination, suppose HPLC grade water shouldn't have any organic compounds. In some research centre, the water will be purified using a combination of methods, such as reverse osmosis membranes, ion-exchange polymer, activated carbon, ultraviolet rays, and ultrafiltration. It is no doubt that the water quality bring serious impact to the HPLC test of all 5 sample and distilled water.

After reviewing all of the problem in chromatogram, it is suspect that the reason leading to all these abnormal in chromatogram is mobile phase and column, especially column. The possible cause for the column problem is column contamination or column degradation. The column used is C-18 column, it is well-known as best preserved in high organic solvents. This column has been using for very long time, it is suspect that simply purging of the column is insufficient for column cleaning. Acetonitrile is a stronger solvent than methanol and is suitable use to clean the column. It is suggested to clean the column with isocratic H20/CAN (70/30) to wash salts, then follow by washing it with isocratic H20/CAN (30/70) to keep your column high organic until next use. It is suggested to clean the column following manual and if the problem is remaining after cleaning, replace a new column is highly recommended. Beside the sample, the distilled water also tested using HPLC as blank.

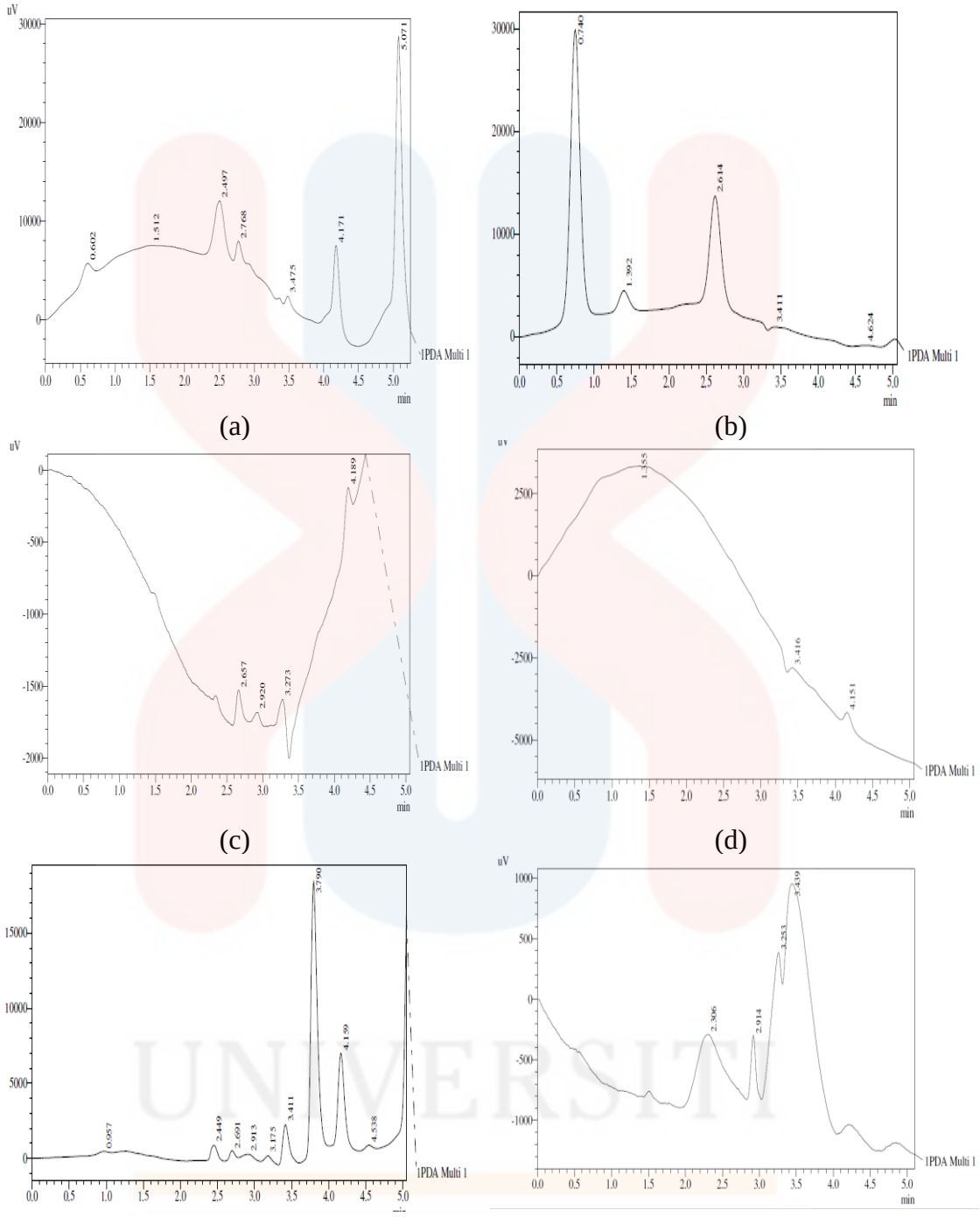

Figure 4.13: The chromatogram of (a) sample 1 produced using bioreactor with pH 5, (b) sample 2 produced using bioreactor with pH 7, (c) sample 3 produced using shaker with pH 5, (d) sample 4 produced using shaker with pH 6, (e) sample 5 produced using shaker with pH 8, (f) distilled water

Table 4.7: Detail information for each peak in figure 4.13 (a).

Peak #	Retention time	area	height	Area%	Height %
1	0.602	124264	5767	8.349	7.817
2	1.512	647112	7647	43.480	10.366
3	2.497	220741	12241	14.832	16.592
4	2.768	202805	8200	13.627	11.115
5	3.475	28350	2668	1.905	3.617
6	4.171	68697	9004	4.616	12.205
7	5.071	196320	28246	13.191	38.288
Total		1488289	73772	100.000	100.000

Table 4.8: Detail information for each peak in figure 4.13 (b).

Peak #	Retention time	area	height	Area%	Height %
1	0.740	278758	28803	53.880	65.802
2	1.392	32015	2357	6.188	5.385
3	2.614	195758	12073	37.837	27.581
4	3.411	9454	420	1.827	0.959
5	4.624	1383	120	0.267	0.273
Total		517368	43773	100.000	100.000

Table 4.9: Detail information for each peak in figure 4.13 (c).

Peak #	Retention time	area	height	Area%	Height %
1	2.657	1918	268	20.423	23.806
2	2.920	1831	190	19.492	16.899
3	3.273	4123	383	43.899	33.978
4	4.189	1520	285	16.185	25.317
Total		9392	1127	100.000	100.000

Table 4.10: Detail information for each peak in figure 4.13 (d).

Peak #	Retention time	area	height	Area%	Height %
1	1.355	581025	4480	98.637	88.061
2	3.416	5895	249	1.001249	4.895
3	4.151	2136	358	0.363583	7.044
Total		589056	5088	100.000	100.000

Table 4.11: Detail information for each peak in figure 4.13 (e).

Peak #	Retention time	area	height	Area%	Height %
1	0.957	1248	158	0.612	0.504
2	2.449	6156	1036	3.017	3.314
3	2.691	3925	710	1.923	2.272
4	2.913	5040	471	2.470	1.506
5	3.175	2927	466	1.435	1.491
6	3.411	16048	2655	7.865	8.497
7	3.790	117941	18636	57.800	59.635
8	4.159	48601	6799	23.818	21.756
9	4.538	2166	320	1.062	1.024
Total		204051	31251	100.000	100.000

Table 4.12: Detail information for each peak in figure 4.13 (f).

Peak #	Retention time	area	height	Area%	Height %
1	2.306	18194	644	24.645	13.499
2	2.914	4432	699	5.273	14.661
3	3.253	15444	1419	18.373	29.751
4	3.439	45986	2007	54.709	42.088
Total		589056	5088	100.000	100.000

4.5.2 Monitoring Data of Sample 1 and 2 which is Using Bioreactor for Fermentation

Unfortunately, although there is prepared a calibration curve in figure 4.10, beside the result of figure 4.13 (b) and 4.13 (e), all other chromatogram showed very serious baseline shifting problem that bring negative impact to the result. Although this 2 chromatogram have better baseline among all chromatogram, but there is many peak present. The more peak obtain from the chromatogram, the less pure the sample is obtain. The retention time for ethanol obtain from standard is around 3.3 minutes. The figure 4.13 and 4.17 showed the major component peak appear after 3.3 minutes while the figure 4.14 and figure 4.16 showed the major component peak appear before 3.3 minutes. This showed that the major component obtain in figure 4.13 and figure 4.17 is not ethanol but less polar than ethanol following the principle of polarity, ethanol is less polarity than lactic acid but more polarity than aldehyde or ketone. It is suspect that the major component peak after ethanol peak is aldehyde and ketone while the other two is lactic acid. (E.Ophardt, 2003)

The parameter of the fermentation was pH, in sample 1 and 2 the pH set is 5 and 7. Figure 4.19 (left) and figure 4.22 (left) showed that during both fermentation process the pH is remain constant from the beginning to the end. If comparing figure 4.21 and 4.24, only sample 1 showed anaerobic condition along the fermentation period because there is no air enter the bioreactor, the amount of dissolve oxygen in fermentation broth is near to zero during fermentation. Unfortunately there is some air enter the bioreactor after 2 hours start of fermentation.

The figure 4.19 showed when the system detect the entry of air into the bioreactor; the fermentation was started on 9am morning, the input of air is detect on 11 am, 1 pm and 9 pm. Although the bioreactor is immediately check when found the air is enter bioreactor during 1pm, but at 9pm system still detect the present of air enter bioreactor for a little while. All the used and unused lid port is seal with parafilm to ensure no air enter. Unfortunately, the amount of dissolve oxygen in fermentation broth is increase in the middle of fermentation. The amount of dissolve oxygen is increasing, it is believe that there is other reaction occur that acidified the fermentation broth and produce dissolve oxygen. During the halfway of fermentation that is not in supervision, all the base solution prepared for adjust pH is finish used, but the system wasn't able to detect and keep pumping air inside the base solution bottle into the bioreactor, this last for approximate 1-2 hours until it was found. Although the base solution is refill immediately, but it already bring serious impact to the reaction happen in fermentation broth. From the figure 4.15 (left) and 4.18 (left), it can be found that no acid used by bioreactor system to adjust pH, but from figure 4.15 (right) and figure 4.18 (right) the amount of base used is keep increasing during fermentation. This showed the fermentation process will produce ethanol and others substance that increasing the acidity of the fermentation broth, the lactic acid is produce in the fermentation that used ragi tempeh as fermentation starter, (Millati et al., 2005) hence more base is enter the fermentation broth to ensure the pH stay constant.

From the observation of figure 4.14 to 4.19, there is one change in common, which is the reaction in fermentation broth will acidify the fermentation broth, the used of base solution by system to adjust pH proved this statement. The ethanol is well-known to produce in anaerobic condition, which is absence of oxygen. Since the ragi tempeh is used and the starter culture is rhizopus spp bacteria, with the presence of oxygen the amount

of lactic acid production. Generally, the higher the lactic acid production, the lower the ethanol production. Rhizopus sp requires oxygen supply to carry out aerobic fermentation to produce lactic acid. For pH 5 sample, which is sample 1, since there is no dissolve oxygen enter the fermentation broth, the amount of lactic acid produce is very little, only a few ml of base solution is used to adjust back the pH to 5, the pH value will drop during its production. The condition in pH 7 sample is different, since the dissolve oxygen is enter due to the experimental mistake, the fermentation change to aerobic fermentation for rhizopus sp bacteria. Lactic acid is produce and the amount of ethanol is decrease. From figure 4.15 (right), the use of base solution is keep increase indicate the production of lactic acid, but from figure 4.14 (right) showed the amount of dissolve oxygen is decrease when the fermentation is going to stop. It is predicted as the amount of dissolve oxygen is used up hence the production of lactic acid is slowing down, aerobic fermentation was started to convert back to anaerobic fermentation.

Figure 4.17 to figure 4.19 showed the fermentation data that contribute to the result of figure 4.13 (b). But from figure 4.13 (b) the result of chromatogram still showed low production of ethanol at the end. The retention time at 0.74 minutes showed major peak area and is suspect to be lactic acid. Lactic acid is a polar molecule, according to the principle of polarity of organic compound, the polarity of acid is greater than alcohol (E.Ophardt, 2003). From figure 4.13 (b) the major component peak appear before retention time of ethanol showing this major component is more polar than ethanol, Although there is no testing to prove the present of lactic acid in sample, but it is highly suspected to be lactic acid.

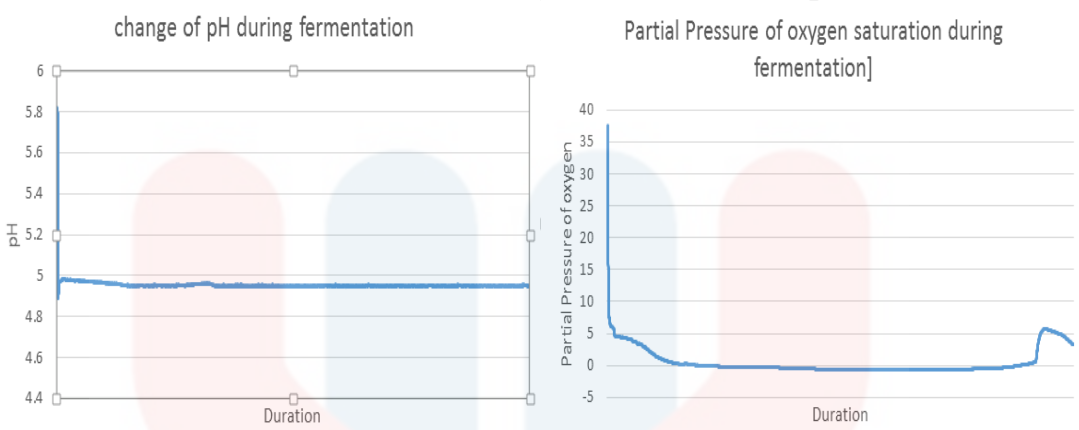

Figure 4.14: The monitor of pH during fermentation using bioreactor (left), the monitor of dissolve oxygen amount during fermentation using bioreactor. (Right).

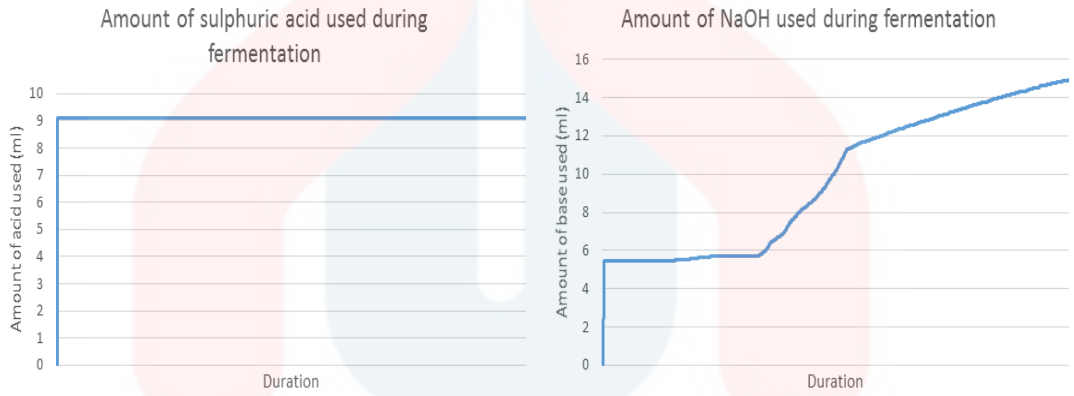

Figure 4.15: The monitor of the used of acid (left) and base (right) during fermentation using bioreactor to constant the pH of fermentation broth to 5.

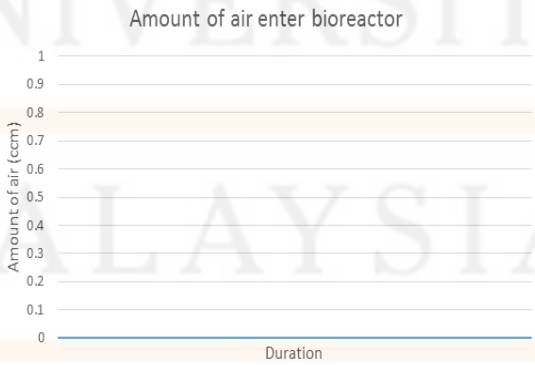

Figure 4.16: The monitor of the amount of air enter the bioreactor during fermentation.

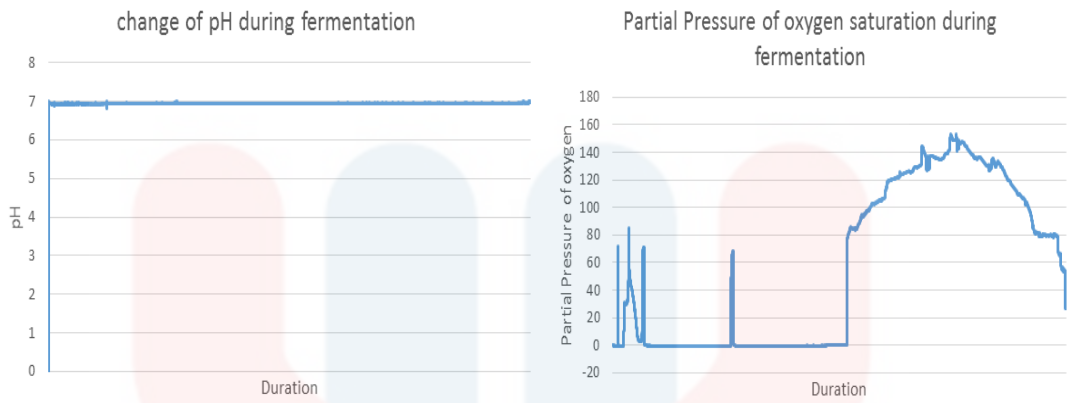

Figure 4.17: The monitor of pH during fermentation using bioreactor (left), the monitor of dissolve oxygen amount during fermentation using bioreactor. (Right).

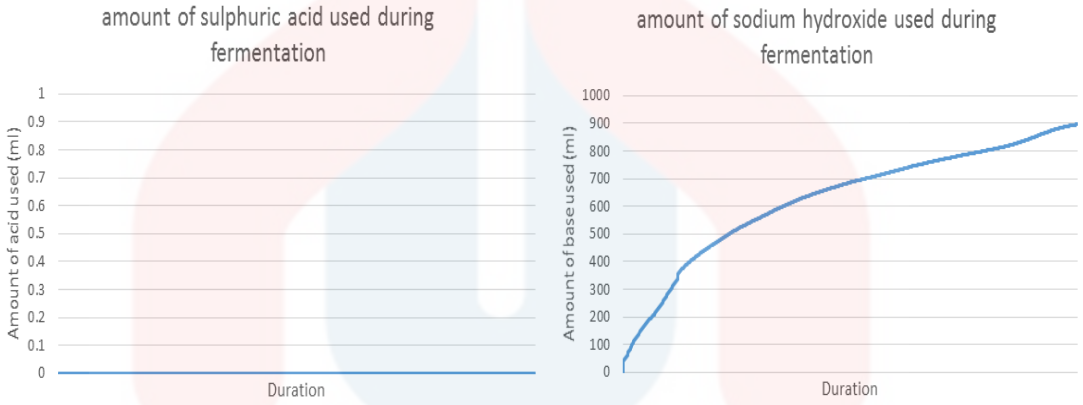

Figure 4.18: The monitor of the used of acid (left) and base (right) during fermentation using bioreactor to constant the pH of fermentation broth to 7.

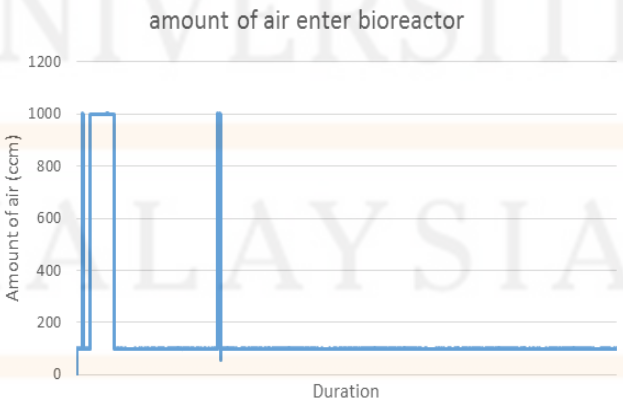

Figure 4.19: The monitor of the amount of air enter the bioreactor during fermentation.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

The bioethanol production from lignocellulosic biomass such as tropical grass is recommended because it is cheap, and able to found in everywhere, but the proper pretreatment and enzymatic hydrolysis to the feedstock is challenging and costly. The chemical pretreatment is necessary for the degradation of lignocellulosic feed stock for better release of fermentation substrate and ease of enzymatic hydrolysis. Besides the use of chemical substance, the microorganism is involve to utilize the fermentable substrate to bioethanol. This paper belongs to lab scale study, the test used for characterisation is freezing test, Tollens' test, Fourier Transform Infrared Spectrometer (FTIR), and High Performance Liquid Chromatography (HPLC).

The concentration of acid used in pretreatment, pH of fermentation broth, and fermentation using bioreactor and shaker, is the parameter selected for this study, the concentration of acid for pretreatment is 0.6%, 0.9%, 1.2%, 1.5%, 5.0% and 15%. Although 15% sulphuric acid pretreatment showed the greater amount of glucose release, but the characterisation test showed low ethanol concentration in all sample. Among all HPLC chromatogram, the sample obtained from pH 8 fermentation broth using shaker

showed the highest ethanol yield compare to other which is 7.8% of ethanol. This is not expected because the optimum fermentation pH is within 5-7.

To study the composition of the product, the Tollens' test showed that the aldehyde is present in all the sample after fermentation, the aldehyde is inhibitory substance that cause the inhibitory of activity of microorganisms, lower the yield of substrate be consumed and fermented to ethanol. The FTIR test showed the sample recovered from fermentation broth most likely to be water, further analysis using HPLC showed the ethanol concentration is very low in all sample.

5.2 Recommendation

Regardless of the problem related to chromatogram and HPLC, the FTIR detection showed the sample collected from fermentation broth is highly similar with water, fermentation using ragi tempeh as starter has low bioethanol yield. It is highly recommend to create an anaerobic condition and minimize dissolve oxygen content in fermentation broth, the present of oxygen that trigger aerobic respiration will product more lactic acid than ethanol. The use of shaker for anaerobic fermentation is not recommendable because it is hard to sustain the anaerobic environment in fermentation vessel.

The acid pretreatment process will produce inhibitory substance that bring inhibitory effect to microorganisms or enzymatic activity. Due to the limitation during laboratory work, the characterisation for pretreated feedstock sample isn't carry out to identify its component before proceed to fermentation. If the component can be identify, the proper use of enzyme and correction measure can be carry out before proceed to

fermentation, this can save a lot of time and resource to raise the bioethanol yield from fermentation.

The most significant problem showed in result and discussion is the irregular chromatogram result. For detection of ethanol using HPLC, it is suggest to use Refractive index (RI) detector, the UV detector such as PDA used in this study is less appropriate for HPLC ethanol detection, it required indirect method and use of formic acid as mobile phase, and this is actually the very old method for HPLC ethanol detection and analysis. Beside the reason of HPLC component, the column contamination is another problem for inaccurate data obtain in chromatogram. Purging with mobile phase is not sufficient for cleaning column.

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UNIVERSITI MALAYSIA KELANTAN

HPLC CHROMATOGRAM

Sample Information

Date : 2018-11-22
Day : Thu
Time : 1015

Acquired by	: Admin
Sample Name	: ethanol
Sample ID	: 25%
Tray#	: 1
Vail#	: 1
Injection Volume	: 20 uL
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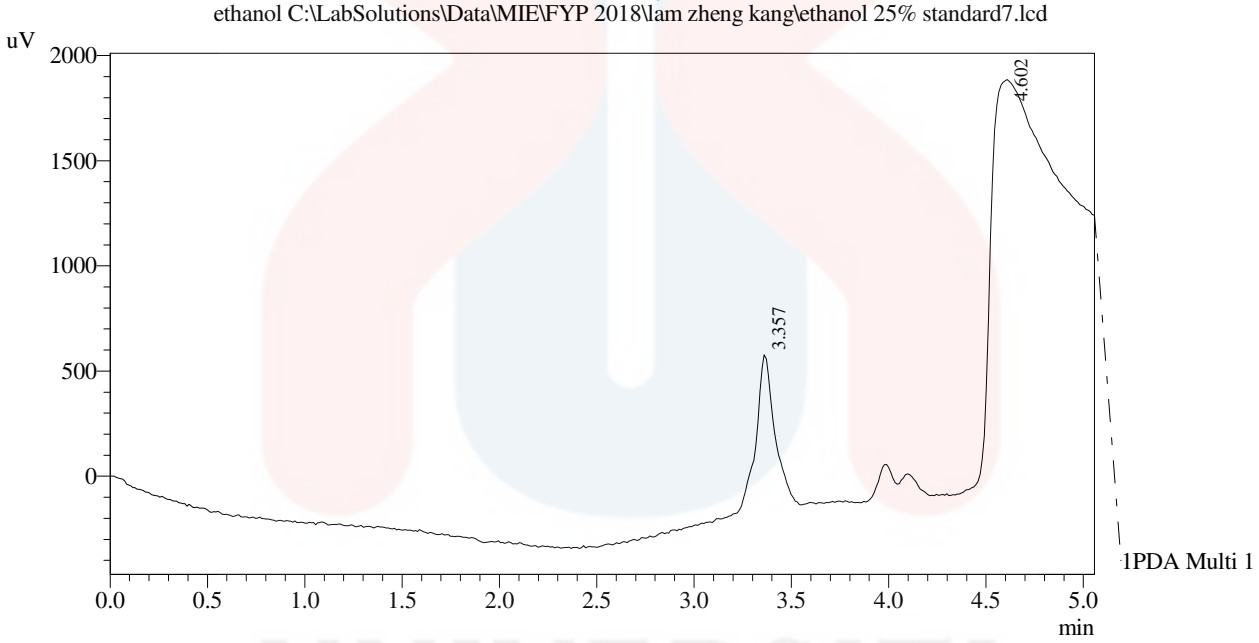

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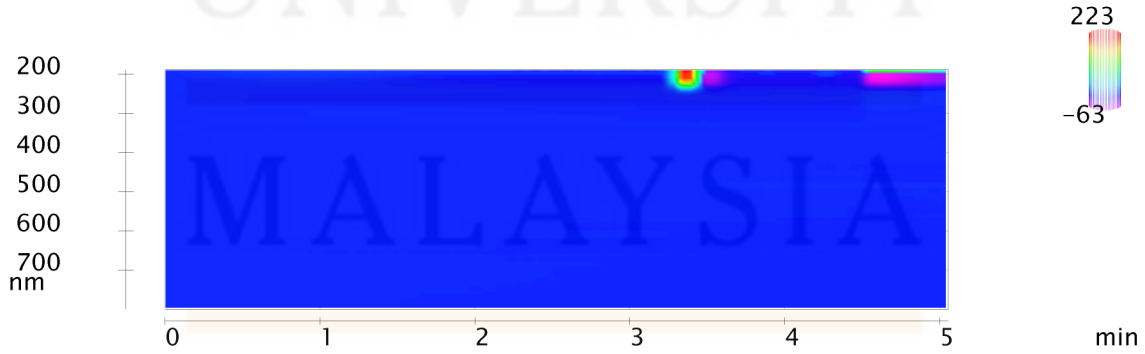

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HPLC CHROMATOGRAM

Sample Information

Date : 2018-11-21
Day : Wed
Time : 1711

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Sample Name	: ethanol
Sample ID	: 50%
Tray#	: 1
Vail#	: 2
Injection Volume	: 20 uL
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Method Filename	: Ethanol Zaleha.lcm
Batch Filename	: quercetin mie.lcb
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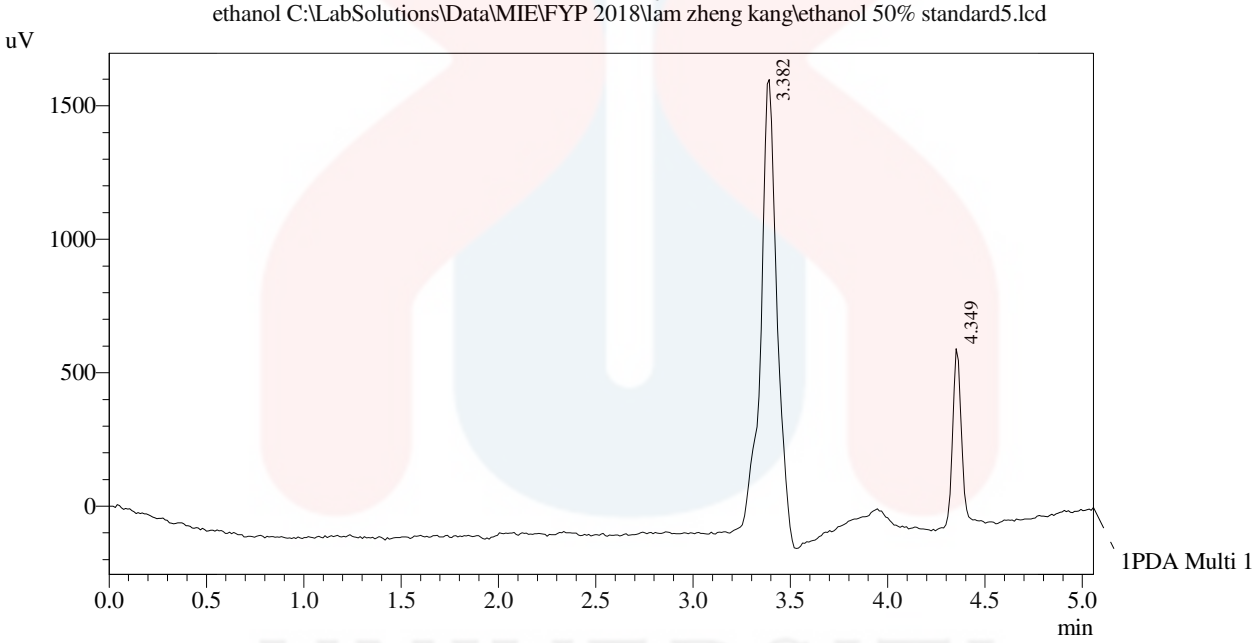

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Contour

PeakTable

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2	4.349	2073	664	16.885	27.763
Total		12279	2392	100.000	100.000

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HPLC CHROMATOGRAM

Sample Information

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Day : Wed
Time : 1718

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Sample ID	: 75%
Tray#	: 1
Vail#	: 3
Injection Volume	: 20 uL
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Batch Filename	: quercetin mie.lcb
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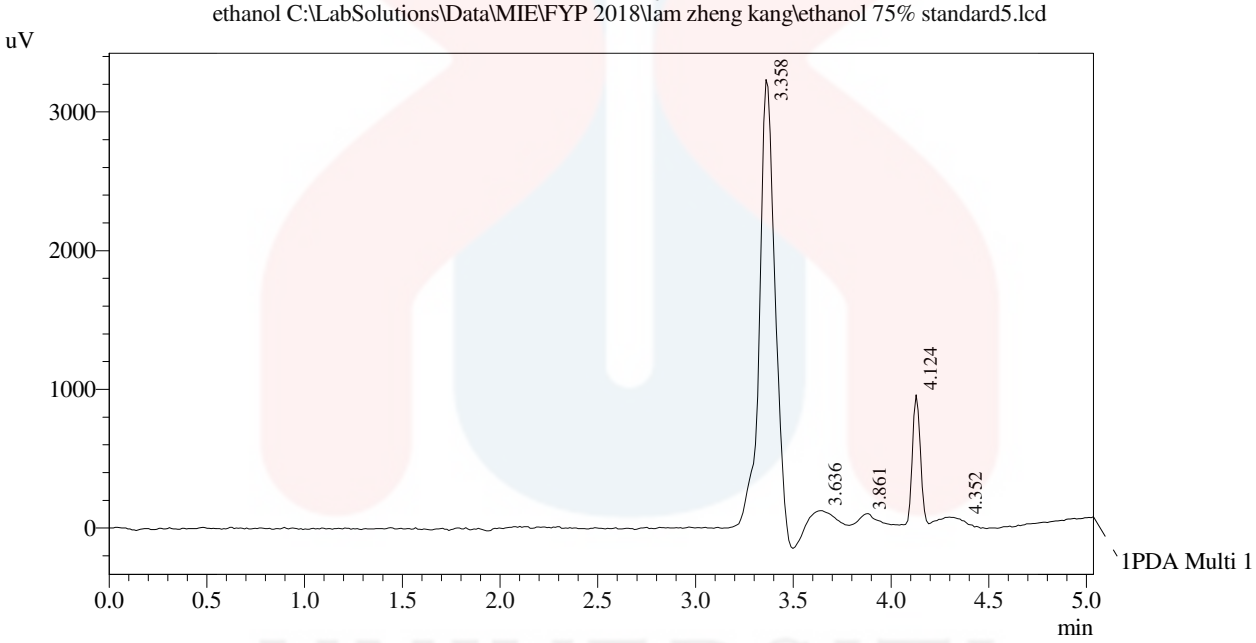

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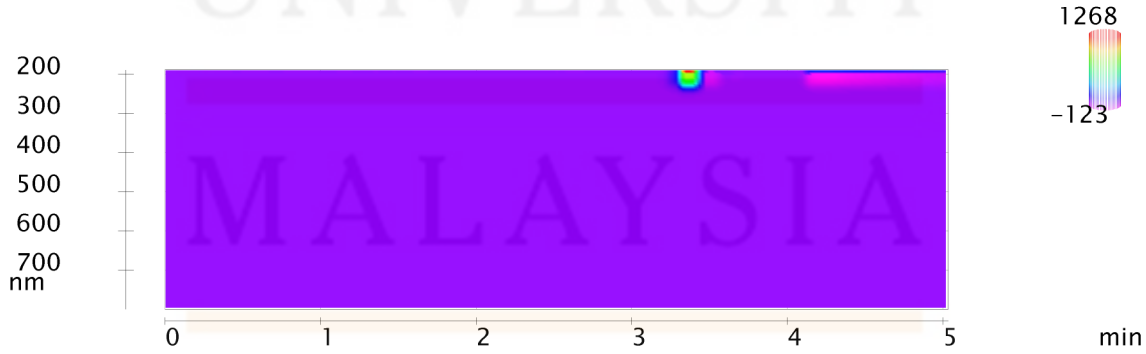

PeakTable

PDA Ch1 254nm 4nm

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1	3.358	18891	3318	66.631	68.439
2	3.636	2965	252	10.457	5.192
3	3.861	2233	187	7.877	3.853

Peak#	Ret. Time	Area	Height	Area %	Height %
4	4.124	3168	1011	11.176	20.853
5	4.352	1094	81	3.860	1.664
Total		28352	4848	100.000	100.000

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HPLC CHROMATOGRAM

Sample Information

Date : 2018-11-21
Day : Wed
Time : 1724

Acquired by	: Admin
Sample Name	: ethanol
Sample ID	: 100%
Tray#	: 1
Vail#	: 4
Injection Volume	: 20 uL
Data Filename	: ethanol 100% standard5.lcd
Method Filename	: Ethanol Zaleha.lcm
Batch Filename	: quercetin mie.lcb
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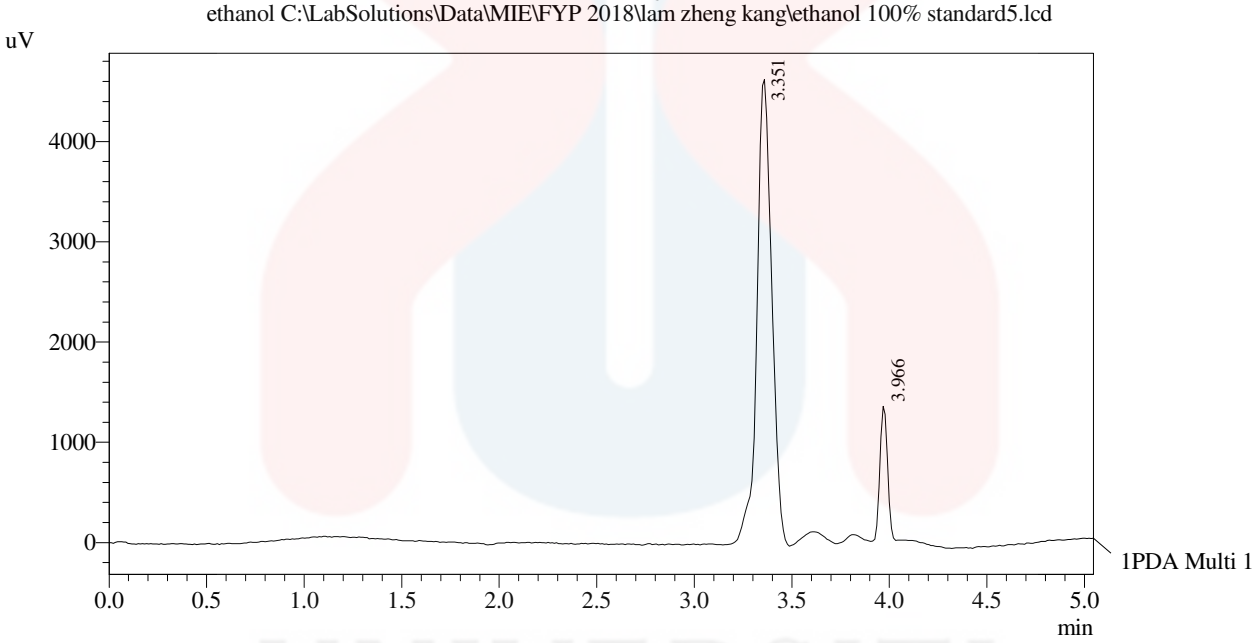

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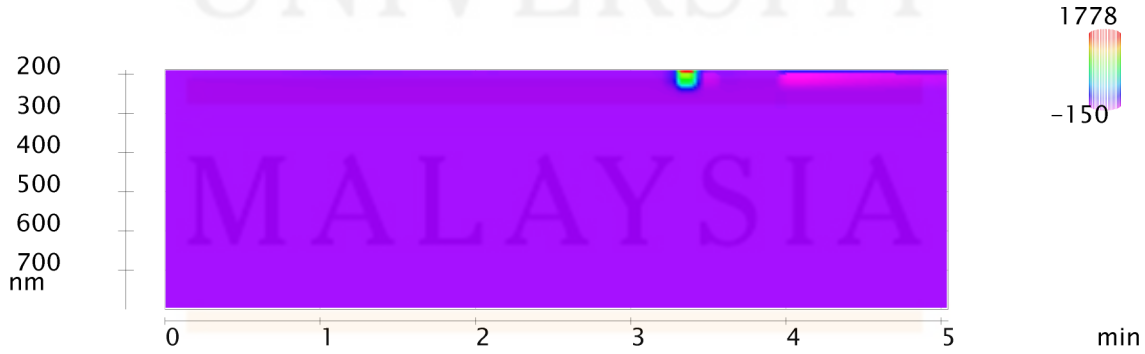

PeakTable

PDA Ch1 254nm 4nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.351	24776	4650	87.065	77.574
2	3.966	3681	1344	12.935	22.426
Total		28457	5994	100.000	100.000

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HPLC CHROMATOGRAM

Sample Information

Date : 2018-11-22
Day : Thu
Time : 1127

Acquired by	: Admin
Sample Name	: ethanol
Sample ID	: reactor, pH5
Tray#	: 1
Vail#	: 8
Injection Volume	: 20 uL
Data Filename	: pH5R1.lcd
Method Filename	: Ethanol Zaleha.lcm
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Chromatogram
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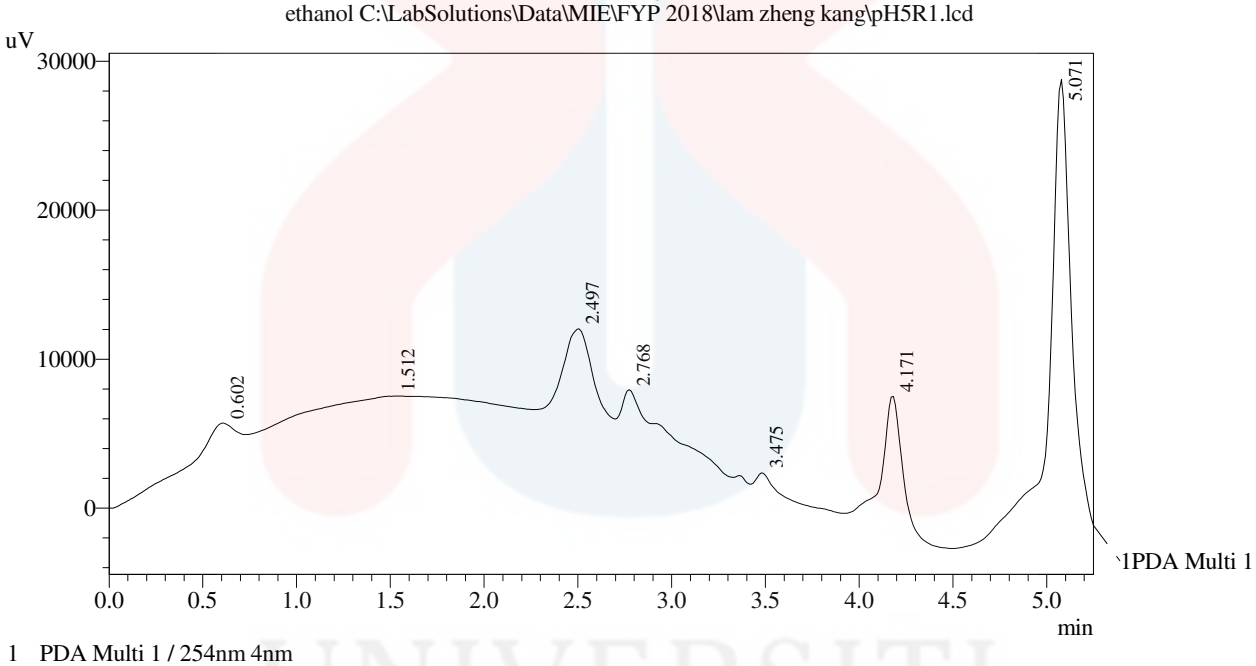

1 PDA Multi 1 / 254nm 4nm

Contour

PeakTable

PDA Ch1 254nm 4nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	0.602	124264	5767	8.349	7.817
2	1.512	647112	7647	43.480	10.366
3	2.497	220741	12241	14.832	16.592

Peak#	Ret. Time	Area	Height	Area %	Height %
4	2.768	202805	8200	13.627	11.115
5	3.475	28350	2668	1.905	3.617
6	4.171	68697	9004	4.616	12.205
7	5.071	196320	28246	13.191	38.288
Total		1488289	73772	100.000	100.000

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HPLC CHROMATOGRAM

Sample Information

Date : 2018-11-22
Day : Thu
Time : 1150

Acquired by	: Admin
Sample Name	: ethanol
Sample ID	: reactor, pH7
Tray#	: 1
Vail#	: 9
Injection Volume	: 20 uL
Data Filename	: pH7R1.lcd
Method Filename	: Ethanol Zaleha.lcm
Batch Filename	: quercetin mie.lcb
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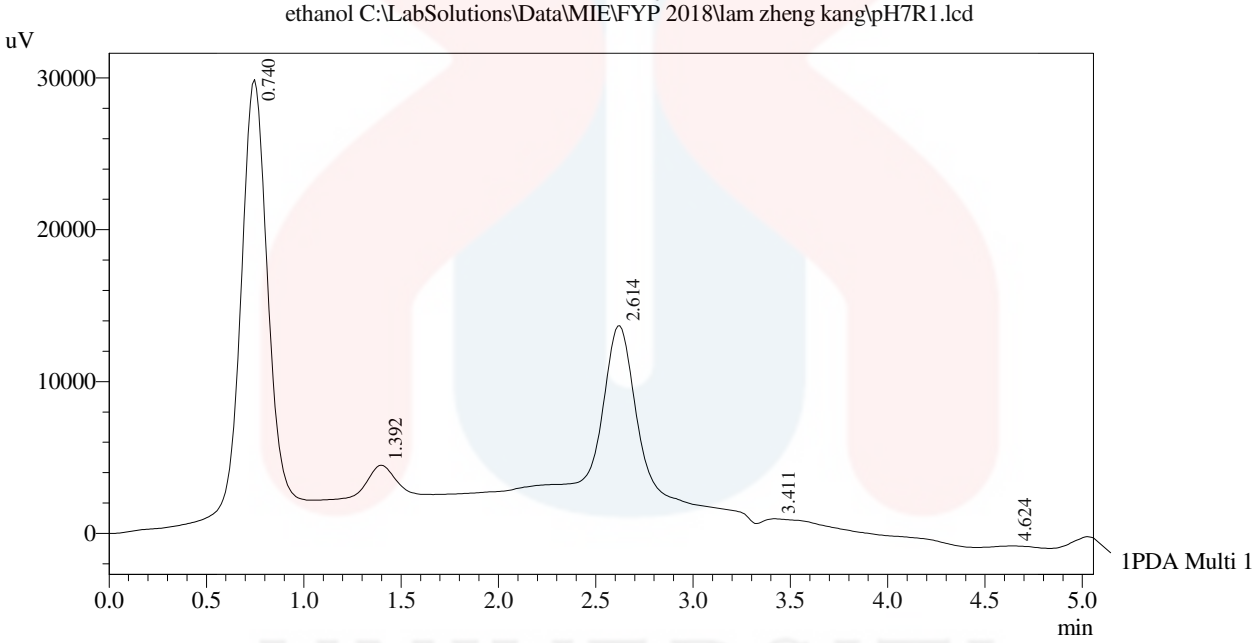
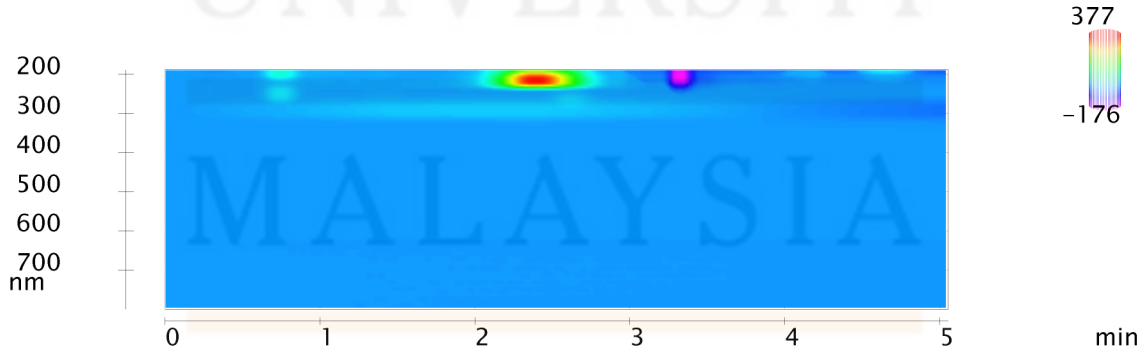
Chromatogram
ethanol C:\LabSolutions\Data\MIE\FYP 2018\lam zheng kang\pH7R1.lcd



1 PDA Multi 1 / 254nm 4nm

Contour



PeakTable

PDA Ch1 254nm 4nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	0.740	278758	28803	53.880	65.802
2	1.392	32015	2357	6.188	5.385
3	2.614	195758	12073	37.837	27.581

Peak#	Ret. Time	Area	Height	Area %	Height %
4	3.411	9454	420	1.827	0.959
5	4.624	1383	120	0.267	0.273
Total		517368	43773	100.000	100.000

UNIVERSITI MALAYSIA KELANTAN

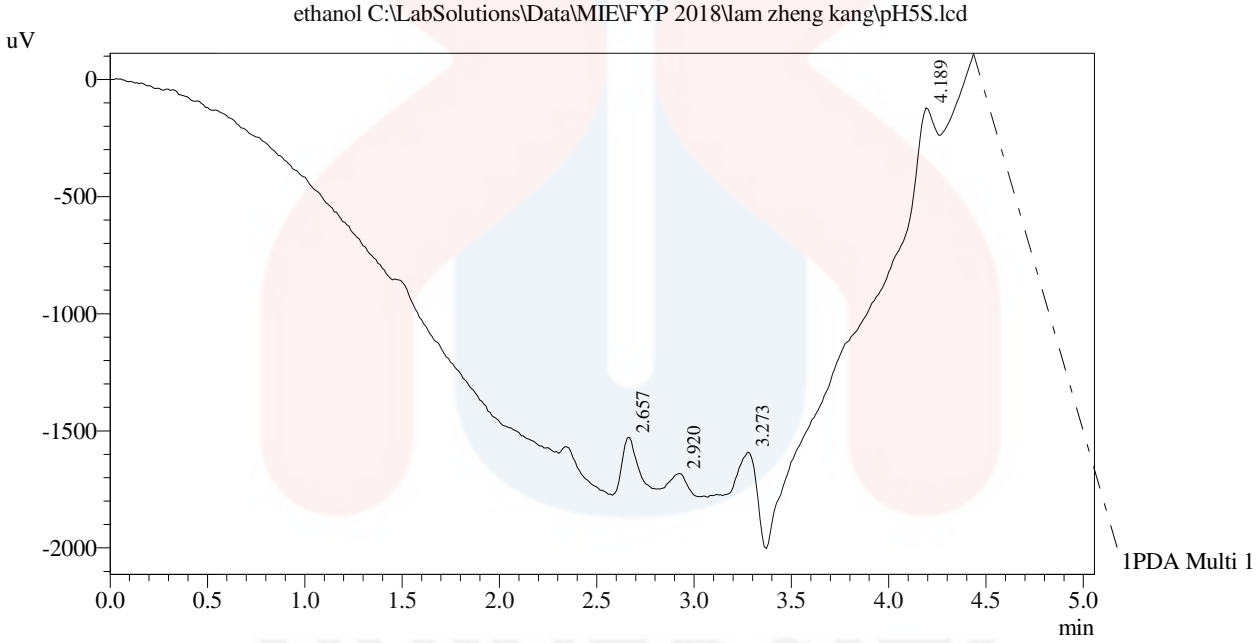
HPLC CHROMATOGRAM

Sample Information

Date : 2018-11-22
Day : Thu
Time : 1151

Acquired by	: Admin
Sample Name	: ethanol
Sample ID	: shaker, pH5
Tray#	: 1
Vail#	: 5
Injection Volume	: 20 uL
Data Filename	: pH5S.lcd
Method Filename	: Ethanol Zaleha.lcm
Batch Filename	: quercetin mie.lcb
Report Filename	: Izmer_format_07082015.lcr
Date Acquired	: 11/22/2018 10:38:50 AM
Data Processed	: 11/22/2018 10:43:55 AM

Chromatogram
ethanol C:\LabSolutions\Data\MIE\FYP 2018\lam zheng kang\pH5S.lcd



1 PDA Multi 1 / 254nm 4nm

Contour

PeakTable

PDA Ch1 254nm 4nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.657	1918	268	20.423	23.806
2	2.920	1831	190	19.492	16.899
3	3.273	4123	383	43.899	33.978

Peak#	Ret. Time	Area	Height	Area %	Height %
4	4.189	1520	285	16.185	25.317
Total		9392	1127	100.000	100.000

UNIVERSITI MALAYSIA KELANTAN

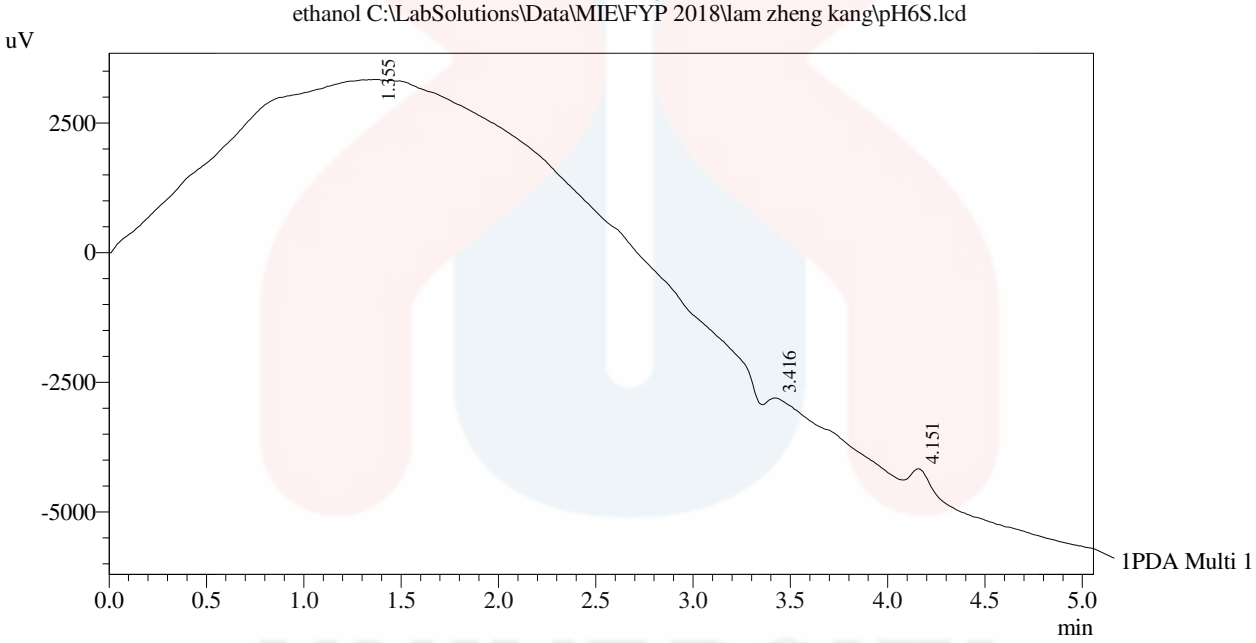
HPLC CHROMATOGRAM

Sample Information

Date : 2018-11-22
Day : Thu
Time : 1109

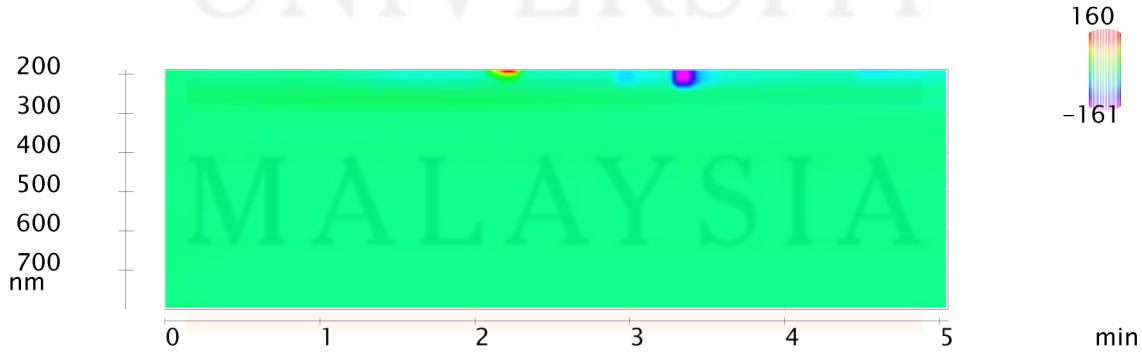
Acquired by	: Admin
Sample Name	: ethanol
Sample ID	: shaker, pH6
Tray#	: 1
Vail#	: 6
Injection Volume	: 20 uL
Data Filename	: pH6S.lcd
Method Filename	: Ethanol Zaleha.lcm
Batch Filename	: quercetin mie.lcb
Report Filename	: Izmer_format_07082015.lcr
Date Acquired	: 11/22/2018 11:04:33 AM
Data Processed	: 11/22/2018 11:09:38 AM

Chromatogram
ethanol C:\LabSolutions\Data\MIE\FYP 2018\lam zheng kang\pH6S.lcd



1 PDA Multi 1 / 254nm 4nm

Contour



PeakTable

PDA Ch1 254nm 4nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	1.355	581025	4480	98.637	88.061
2	3.416	5895	249	1.001	4.895
3	4.151	2136	358	0.363	7.044

Peak#	Ret. Time	Area	Height	Area %	Height %
Total		589055	5088	100.000	100.000

UNIVERSITI MALAYSIA KELANTAN

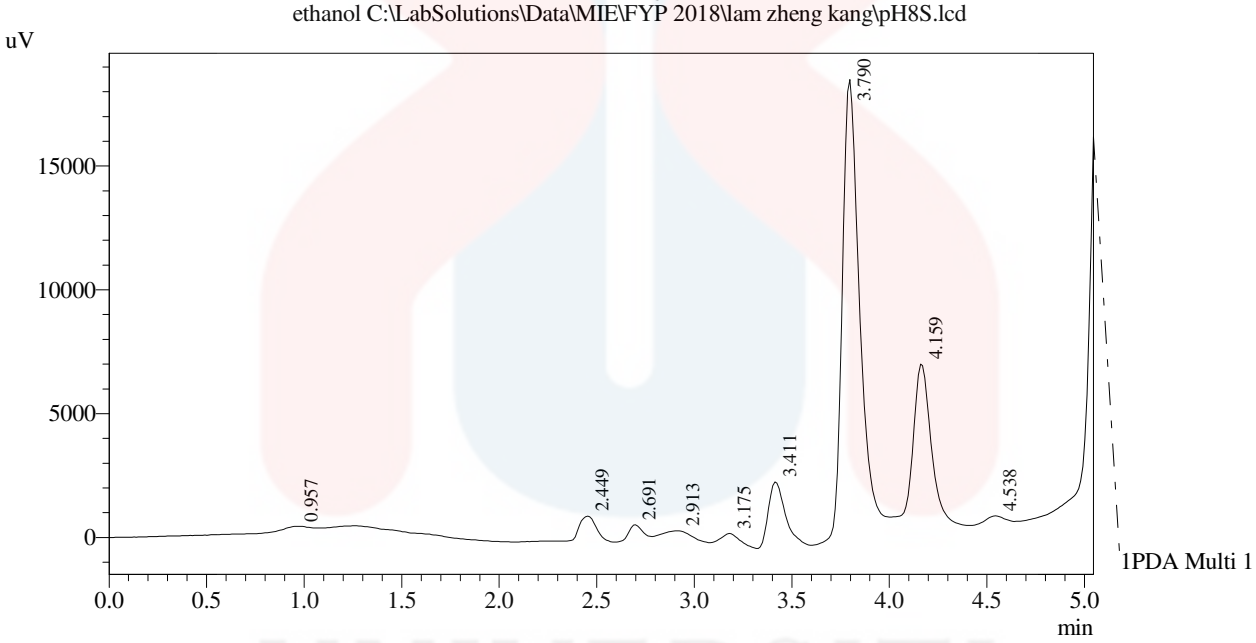
HPLC CHROMATOGRAM

Sample Information

Date : 2018-11-22
Day : Thu
Time : 1118

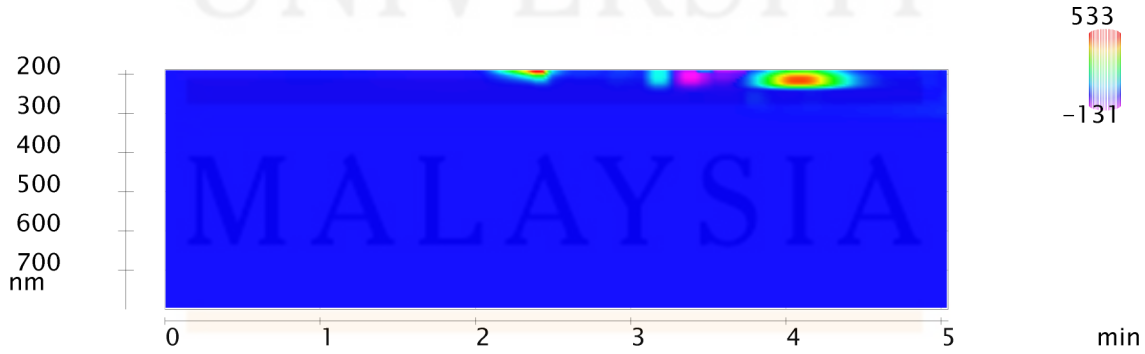
Acquired by	: Admin
Sample Name	: ethanol
Sample ID	: shaker, pH8
Tray#	: 1
Vail#	: 7
Injection Volume	: 20 uL
Data Filename	: pH8S.lcd
Method Filename	: Ethanol Zaleha.lcm
Batch Filename	: quercetin mie.lcb
Report Filename	: Izmer_format_07082015.lcr
Date Acquired	: 11/22/2018 11:13:16 AM
Data Processed	: 11/22/2018 11:18:20 AM

Chromatogram
ethanol C:\LabSolutions\Data\MIE\FYP 2018\lam zheng kang\pH8S.lcd



1 PDA Multi 1 / 254nm 4nm

Contour



PeakTable

PDA Ch1 254nm 4nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	0.957	1248	158	0.612	0.504
2	2.449	6156	1036	3.017	3.314
3	2.691	3925	710	1.923	2.272

Peak#	Ret. Time	Area	Height	Area %	Height %
4	2.913	5040	471	2.470	1.506
5	3.175	2927	466	1.435	1.491
6	3.411	16048	2655	7.865	8.497
7	3.790	117941	18636	57.800	59.635
8	4.159	48601	6799	23.818	21.756
9	4.538	2166	320	1.062	1.024
Total		204051	31251	100.000	100.000

UNIVERSITI MALAYSIA KELANTAN

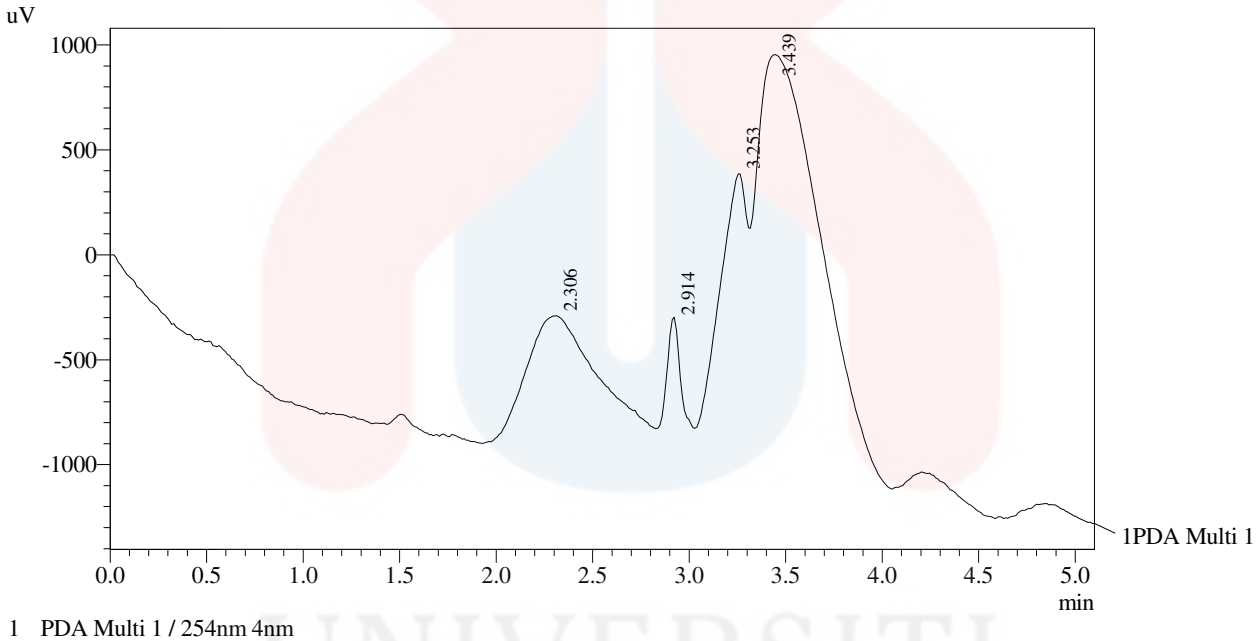
HPLC CHROMATOGRAM

Sample Information

Date : 2018-11-22
Day : Thu
Time : 1146

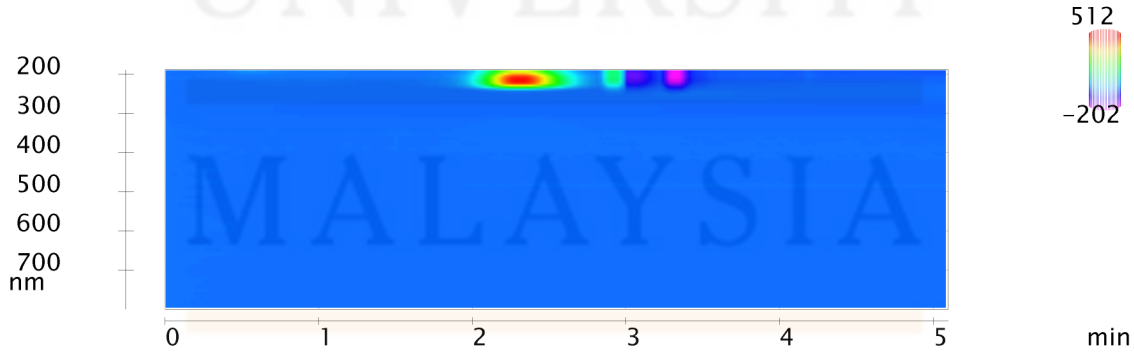
Acquired by	: Admin
Sample Name	: water
Sample ID	: 6
Tray#	: 1
Vail#	: 12
Injection Volume	: 20 uL
Data Filename	: water3.lcd
Method Filename	: Ethanol Zaleha.lcm
Batch Filename	: quercetin mie.lcb
Report Filename	: Izmer_format_07082015.lcr
Date Acquired	: 11/22/2018 11:41:18 AM
Data Processed	: 11/22/2018 11:46:25 AM

Chromatogram
water C:\LabSolutions\Data\MIE\FYP 2018\zaleha\water3.lcd



1 PDA Multi 1 / 254nm 4nm

Contour



PeakTable

PDA Ch1 254nm 4nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.306	18194	644	21.645	13.499
2	2.914	4432	699	5.273	14.661
3	3.253	15444	1419	18.373	29.751

Peak#	Ret. Time	Area	Height	Area %	Height %
4	3.439	45986	2007	54.709	42.088
Total		84056	4768	100.000	100.000